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CHARACTERIZATION OF CARBOXYLESTERASES INVOLVED
IN THE INSECTICIDE RESISTANCE OF
CULEX QUINQUEFASCIATUS
FROM THE CARIBBEAN AND SOUTH AMERICA

BY

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A thesis submitted for the degree of Doctor of Philosophy
DEPARTMENT OF MEDICAL PARASITOLOGY
LONDON SCHOOL OF HYGIENE AND TROPICAL MEDICINE
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1996



ACKNOWLEDGEMENTS

I wish to express my sincere thanks to my supervisor Professor Janet Hemingway for giving me the opportunity of embarking on this study and for her guidance and encouragement throughout its course.

I would also like to thank Dr. S.H.P. Parakarama Karunaratne for his friendship, helpful advice, and assistance in the characterization of the Colombia and Trinidad EstB1 esterases. I am also grateful to Juan Bisset and Magdalena Rodriguez at the Instituto Tropical Medicino 'Pedro Kouri', Havana, Cuba for help in the collection and initial screening of populations for insecticide resistance, and Dr. Chris Curtis for providing the Colombia strain.

My sincere thanks also to Dr. K.G.I. Jayawardena, Dr. La-aied Prapanthadara, Dr. Ashley Vaughan, Hilary Ranson, Barabara Sawyer, Tracey Joseph and many more at the London School of Hygiene and Tropical Medicine for their friendship throughout my stay.

Finally, I owe my deepest gratitude to my wife Kem, my mother and father, and all the rest of my family for their unstinting support and encouragement. This thesis is dedicated to you.

This study was funded by the Wellcome Trust.

ABSTRACT

The organophosphate resistance-associated elevated esterases Est α 2, Est β 1 and Est β 2 were purified to homogeneity from larvae of the Cuban Habana strain. The bimolecular rate constants (k_a s) of Habana Est β 1 with a range of organophosphates were not significantly different to those of PelRR Est β 2¹, and were higher with some organophosphates than PelRR Est α 2¹ (Karunaratne *et al.*, 1993). The relative insecticide binding efficiency of these esterases could not, therefore, explain why co-amplified *est* α 2 and *est* β 2 are out competing *est* β 1 in the field. On the basis of their k_a s, both Habana Est α 2 and Est β 2 could be distinguished from their equivalents purified from other strains.

In two organophosphate resistant strains of *Culex quinquefasciatus* from Colombia and Trinidad, possessing the amplified esterase genes *est* α 3 and *est* β 1, the *Eco*RI restriction fragment lengths of the *est* β 1 genes and their flanking regions were different both to each other and to those previously reported for TEM-R *est* β 1¹ (Raymond *et al.*, 1991) and MRES *est* β 1² (Vaughan *et al.*, 1995). There were a number of significant differences between the k_a s of purified Colombia, Trinidad and Habana Est β 1s. The low k_a s and high k_3 s for the interaction of Colombia Est β 1 with several insecticides confirmed that, as for Est α 2¹ and Est β 2¹, the main role of Est β 1 is sequestration. The k_a s of Habana, Colombia and Trinidad Est β 1s were higher than that of the electrophoretically identical Est β 1³ purified from the susceptible PelSS strain (Karunaratne *et al.*, 1995a). This suggests that the elevated esterase-based mechanism confers resistance through amplification of alleles coding for esterases having a higher reactivity with the insecticides they sequester than

esterases coded for by their non-amplified counterparts.

A PelRR Est α 2¹ antiserum had the same cross-reactivity with Habana Est α 2 as with Est α 2¹. However, both Habana Est β 1 and Est β 2 had a cross-reactivity of approximately 150-fold less than the Est α 2s.

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CHAPTER 1

Chapter 1

GENERAL INTRODUCTION

1.1. HISTORICAL BACKGROUND

Whether by their direct effect on man as nuisance species, vectors of disease, or by their indirect effect by attacking domestic livestock, crops or possessions, insects have been the subject of attempts to control their number over many centuries. A variety of different methods of control have been developed; chemical (insecticides, pheromones, repellents, attractants, growth regulators), microbial, biological, cultural, physical and genetical. Over the last forty years the concept of integrated pest management (IPM) strategies has been propounded, a general definition of which is "a control strategy in which a number of methods are combined to give stable long-term pest control" (Burn *et al.*, 1987). To date, IPM has been applied successfully to the control of agricultural and horticultural pests but has found little favour with those involved in the development and operation of control campaigns of insect vectors of human disease.

The history of pesticide use dates back many centuries, certainly to before 1000 BC when it was mentioned by Homer. However, it was not until the latter part of the 19th century that use of pesticides became common, and then mainly for the control of agricultural pests. The first documented case of pesticide resistance was for San Jose scale against lime-sulphur in Washington, U.S.A., in 1908 (Melander, 1914).

This was followed by a gradual increase in the number of reported cases of resistance until 1946 when the first of the organochlorine insecticides, DDT, was introduced. From this date to the present, the increasing use of a wide range of synthetic insecticides has been paralleled by an even larger increase in the number of cases of resistance. Indeed, by 1980 resistance had been detected in populations of at least 428 species representing 14 orders and 18 families of insects and acarines (Forgash, 1984). Many of the insect pest species have shown a great genetic flexibility giving rise to a number of different insecticide resistance mechanisms. This, coupled with the very short generation times of these insects and with the application during control campaigns of a single insecticide for long periods and over large areas, explains the rapid selection of these mechanisms of resistance.

As well as being a biting nuisance worldwide, some mosquito species are of major vectorial importance in the transmission of human parasitic and viral diseases such as malaria, filariasis, yellow fever, dengue and encephalitis. For example, today it is estimated that over 250 million people are effected by malaria (WHO, 1992) and over 90 million by lymphatic filariasis (Becker & Rettich, 1993). Despite extensive use of insecticides the number of people effected by vector-borne diseases continue to increase. This, in part, has been due to the numbers and geographical range of insecticide-resistant mosquito species. The first reported case of insecticide resistance in a mosquito species was in 1947 when populations of the salt marsh mosquitoes *Aedes taeniorhynchus* and *Ae. sollicitans* in Florida showed resistance to DDT. The appearance of resistance in other species has been rapid. By 1992, 56 species of Anopheline mosquitoes and 46 species of Culicine mosquitoes had been

For a general review of insecticides and their target sites see Corbett *et al.* (1984).

reported as being resistant to at least one insecticide (WHO, 1992). The question now is: How we can halt this trend? Or better still: How can we reverse the trend and return populations of mosquitoes previously resistant to a sensitivity equivalent to that seen in susceptible populations? Certainly, the possibilities of IPM strategies could and should be explored. However, because of the logistics of developing these strategies, their planning and operation has to be viewed in the long term. In the longer term also is the possibility of rational insecticide design; the design of insecticides that can by-pass or nullify existing resistance mechanisms. Central to this is an understanding of the mechanisms of resistance themselves. In the short term, we are restricted to the use of existing insecticides and those currently being brought onto the market. Here too, the understanding of underlying mechanisms of resistance will be essential in developing resistance management strategies that will extend the useful life of these insecticides by avoiding selection of resistance to them.

1.2. INSECTICIDES AND THEIR TARGET SITES

Early insecticides were of three main types. Firstly, there were the contact insecticides. Best known of these are pyrethrum (extracted from the flower of the plant *Chrysanthemum cinerariaefolium* flowers containing pyrethrins and cinerins) and rotenone (an extract from the root of the plant *Derris- elliptica*). These insecticides are ephemeral, having an active life of hours or days. After penetrating the cuticle of insects, pyrethrum has its insecticidal effect by binding to Na⁺ channel proteins, thus preventing the channel from closing. This causes multiple

discharges of the neuron causing tetanic paralysis and, eventually, death. Rotenone appears to have its major effect by inhibiting L-glutamate oxidation which is followed by blockage of nerve conduction. Both of these insecticides are still used today in agriculture and horticulture. Included in this group also are the fumigant insecticides which, when burnt, produced a toxic smoke. The best known of these is nicotine which mimics the action of acetylcholine at the synaptic junction in insects, binding to the acetylcholine receptor and influencing the acetylcholine-induced increases in Na^+ and K^+ conductance. The second type of early insecticides were the oils which were mainly used on dormant plants to suffocate insects and mites (and their eggs) but were also used as mosquito larvicides, suffocating the larvae when spread over the water surface. The third type were the stomach poisons. These were toxic radicals formulated as salts of metals such as Paris Green (copper-aceto-arsenite), and were used against phytophagous insects.

The advent of the residual contact insecticides came with DDT, the first of what was to become a group of insecticides known as the organochlorines. It was first synthesised in 1874, but its insecticidal properties were not discovered until the 1930s. DDT and its analogues have the same mode of action as pyrethrins and cinerins (see above). Included in the organochlorines also is a sub-group, the cyclodienes, examples of which are aldrin and dieldrin. The cyclodienes bind to the γ -aminobutyric acid (GABA) receptors in the Cl^- channels of neurons preventing Cl^- flux across the nerve membrane. The organochlorines dominated the 1940s and 1950s but, because of their persistence in the environment and their toxicity to non-target organisms, many have been phased out. However, DDT, because of its low

production costs and low toxicity to those applying it, is still widely used in malaria control campaigns.

A second group of residual contact insecticides, the organophosphates (OPs), was first produced in the late 1940s. The target site of the OPs is acetylcholinesterase (AChE) which hydrolyses the neuro-transmitter acetylcholine. Inhibition of AChE, therefore, causes acetylcholine to accumulate at synapses giving constant nervous stimulation resulting in tetanic paralysis. The OPs, because of their lower persistency and greater versatility, were preferred over the organochlorines during the 1960s, and are possibly the most widely used insecticide group in public health pest control today.

A third group of residual contact insecticides, the carbamates (derivatives of carbamic acid), were introduced in 1956 with the compound carbaryl. The persistence of the carbamates and their toxicity generally lie between that of the organochlorines and the organophosphates. The target site of the carbamates, as with the OPs, is AChE.

The most recent group of residual contact insecticides are the pyrethroids. These were produced by modifications of components of natural pyrethrum imparting greater photostability and persistence. The first of these was allethrin which was synthesised in 1949. However, the first pyrethroids to combine high insect toxicity with low mammalian toxicity were introduced in the early 1970s. The pyrethroids, like DDT and its analogues, bind to Na^+ channel proteins of the neuron and

prevent the channel from closing.

1.3. MECHANISMS OF INSECTICIDE RESISTANCE

The mechanisms evolved by insects to resist the effects of insecticides can broadly be classified into four main categories: Behavioural changes, reduced insecticide delivery, target site insensitivity and metabolic resistance.

1.3.1. BEHAVIOURAL RESISTANCE

This is a mechanism whereby an insect avoids or reduces uptake of an insecticide by evolving a behaviour that takes it away from the substrate on which that insecticide is found. It has been reported that the mosquito *Anopheles minimus* has changed its preferred habit of resting indoors to resting outdoors and that this change in behaviour reduces the contact of the mosquito which is sprayed on the inside of houses as part of an anti-malaria control campaign (Bang, 1985). However, the apparent change in resting habit coincided with a greater tendency to feed on domestic cattle suggesting the existence of two or more morphologically undefined species with different host and resting preferences, the species with a preference for man as a host and the indoors of houses as a resting place having been controlled by DDT spraying. This is supported by data suggesting the existence of two species in *An. minimus* in Thailand (Green *et al.*, 1990).

1.3.2 REDUCED INSECTICIDE DELIVERY

Reduced insecticide delivery maybe caused by a thickening of the insect cuticle, a change in the chemical composition of the cuticle, or an increase in excretion of insecticide, all these mechanism reducing the amount of insecticide reaching its target site. Cuticular thickening has been shown in a pyrethroid resistant strain of the house fly, *Musca domestica* (Golenda & Forgash, 1989). Cuticular thickening was also found to be responsible for reduced penetration of DDT, diazinon and other insecticides in the 348 and SKA strains of *M. domestica*, the penetration delaying factor (*pen*) being localised to chromosome III (Sawicki & Lord, 1970). Whilst the lethal dose for these insecticides in the 348 strain (which has only the reduced penetration mechanism) was no larger than in other strains, the reduced penetration mechanism significantly delayed knockdown. In OP resistant strains of the Colorado potato beetle, *Leptinotarsa decemlineata* (Argentine *et al.*, 1994), and the diamondback moth, *Plutella xylostella* (Noppun *et al.*, 1989), resistance was found to be partly due to reduced penetration. In mosquitoes, reduced penetration was found to be partly responsible for OP resistance in a multi-resistant strain of *C. tarsalis* (Apperson & Georghiou, 1975), and in a strain of *C. quinquefasciatus* (Stone & Brown, 1969). There is evidence to suggest that pyrethroid resistance in some field populations of *Heliothis virescens* and *Blattella germanica* is partly due to reduced penetration of the insecticides (Ottea *et al.*, 1995; Bull & Patterson, 1993). Reduced penetration and increased rates of insecticide excretion have been shown to be partly responsible for resistance to the juvenoid methoprene in *M. domestica* (Hammock *et al.*, 1977) whilst, in a strain of *Ae. aegypti*, an increase in the rate of

malathion excretion was found to be responsible for resistance to malathion (Matsumura & Brown, 1961b).

1.3.3. TARGET SITE INSENSITIVITY

In this category of resistance mechanism, an alteration in the target site for an insecticide prevents molecules of that insecticide from interacting with it. Obviously, changes must be such that the target site is not prevented from carrying out its physiological function. In at least two of the examples of target site insensitivity to follow, resistance has been found to be due to the substitution of a single amino acid in the protein sequence of the target site.

1.3.3.1. Altered acetylcholinesterase (AChE) (EC 3.1.1.7)

AChE is the target site for OPs and carbamates (Gepner *et al.*, 1978) which bind with the enzyme to form a phosphorylated or carbamylated complex and prevent AChE from carrying out its normal function of hydrolysing acetylcholine. AChE can usually hydrolyse these insecticides but they are very poor substrates, the regeneration to the active enzyme taking several hours. An alteration in the conformation of this enzyme that reduces OP and carbamate inhibition is a highly effective mechanism of resistance. This mechanism has been reported in a large number of pest species of agricultural and public health importance including several species of mosquito (TABLE 1.1). The selection of this mechanism in field populations of *Culex* has only ever been found in combination with, and preceded

TABLE 1.1. Examples of pests of agricultural and public health importance reported as having an altered AChE-based mechanism of OP and carbamate insecticide resistance.

Pest species	References
The german cockroach, <i>Blattella germanica</i>	(Hemingway <i>et al.</i> , 1993)
The cattle tick, <i>Boophilus microplus</i>	(Nolan <i>et al.</i> , 1972)
The pear bug, <i>Cacopsylla pyri</i>	(Berrada <i>et al.</i> , 1994)
The fruit fly, <i>Drosophila melanogaster</i>	(Pralavorio & Fournier, 1992; Fournier <i>et al.</i> , 1993)
The tobacco bud worm, <i>Heliothis virescens</i>	(Brown & Bryson, 1992)
The two-spotted spider mite, <i>Tetranychus urticae</i>	(Smissaert, 1964)
The green rice leafhopper, <i>Nephotettix cincticeps</i>	(Iwata & Hama, 1972)
The house fly, <i>Musca domestica</i>	(Devonshire, 1975; Oppenoorth <i>et al.</i> , 1977; Moores <i>et al.</i> , 1988)
The peach-potato aphid, <i>Myzus persicae</i>	(Moores <i>et al.</i> , 1994)
The tobacco aphid, <i>M. nicotianae</i>	(Moores <i>et al.</i> , 1994)
The cotton aphid, <i>Aphis gossypii</i>	(Suzuki & Hama, 1994; Silver <i>et al.</i> , 1994)
The citrus thrip, <i>Scirtotrip citri</i>	(Ferrari <i>et al.</i> , 1993)
<i>An. albimanus</i> from Central America	(Georghiou & Pasteur, 1978; Hemingway <i>et al.</i> , 1984)
<i>An. nigerimus</i> from Sri Lanka	(Hemingway <i>et al.</i> , 1986)
<i>An. sacharovi</i> from Turkey	(Hemingway <i>et al.</i> , 1985)
<i>C. quinquefasciatus</i> from Cuba and Tanzania	(Bisset <i>et al.</i> , 1990; Bisset <i>et al.</i> , 1991; Rodriguez <i>et al.</i> , 1993; Khayrandish & Wood, 1993a; Khayrandish & Wood, 1993b)
<i>C. pipiens</i> from Italy and France	(Villani & Hemingway, 1987; Bonning & Hemingway, 1991; Rivet <i>et al.</i> , 1994)
<i>C. tritaeniorhynchus</i> from Japan and Sri Lanka	(Takahashi & Yasutomi, 1987; Hemingway <i>et al.</i> , 1986)

by, an elevated esterase-based mechanism (Villani & Hemingway, 1987; Rodriguez *et al.*, 1993), the two mechanisms having a multiplicative effect on OP and carbamate resistance. In *D. melanogaster*, both the AChE gene and the enzyme itself have been well characterised (Gnagey *et al.*, 1987; Fournier *et al.*, 1992). In an insecticide resistant population of *D. melanogaster*, changes have been seen at both the molecular and translation levels. At the level of translation, an increase in the level of AChE reduces the effects of insecticide inhibition of the enzyme, whilst at the molecular level, a mutant form of the enzyme has been shown to have a reduced inhibition coefficient (k_i) for the insecticides (Fournier & Mutero, 1991; Pralavorio & Fournier, 1992; Fournier *et al.*, 1993). The gene coding for the altered AChE has a point mutation which has led to the substitution of phenylalanine³⁶⁸ for a tyrosine residue. The location of the mutated amino acid lies within the putative active site of the enzyme and data suggests that this is the cause of the reduced insecticide binding affinity of the altered enzyme (Mutero & Fournier, 1992). Other point mutations have also been found in AChE in OP resistant populations of *D. melanogaster* (Phe¹¹⁵ to Ser, Gly³⁰³ to Ala and Ile¹⁹⁹ to Val) which may also be involved with reduced insecticide affinity (Pralavorio & Fournier, 1992).

1.3.3.2. Altered γ -aminobutyric acid (GABA) receptor

GABA receptors are situated in the Cl^- channels of neurons which control Cl^- flux across the nerve membrane and, as the name suggests, they are receptors for the neurotransmitter γ -aminobutyric acid. As mentioned in section 1.2., cyclodienes interact with GABA receptors thus disrupting nerve function. An alteration in

GABA receptors making them insensitive to cyclodienes has been reported in the mosquito *Ae. aegypti* (Thompson *et al.*, 1993), the sweet potato whitefly *Bemisia tabaci* (Anthony *et al.*, 1995), the fruit fly *D. melanogaster* (ffrench-Constant *et al.*, 1992; Steichen & ffrench-Constant, 1994; Bloomquist, 1994), the house fly *M. domestica* (Anthony *et al.*, 1991), the german cockroach *Blattella germanica* (Kadous *et al.*, 1983) and the red flour beetle *Tribolium castaneum* (Lin *et al.*, 1993). Characterisation of the GABA_A receptor-chloride ionophore in vertebrate systems clearly demonstrated that cyclodienes competed with antagonists of the receptor (ffrench-Constant *et al.*, 1991). A similar characterisation of GABA_A receptors in the brain of *M. domestica* showed that cyclodienes interacted with them in the same way (Deng *et al.*, 1991). Comparison of the nucleotide sequences for the GABA receptor gene in a dieldrin (a cyclodiene) resistant strain *Rdl* (ffrench-Constant *et al.*, 1993b) with that in a susceptible strain showed that there was a point mutation leading to an amino acid substitution at position 302 (Ala³⁰² to Ser), in the resistant strain (ffrench-Constant *et al.*, 1993c). A second point mutation was also detected at position 361 (Met³⁶¹ to Ile) although this mutation was not always present in resistant strains (ffrench-Constant *et al.*, 1993c). This part of the protein lies within the second membrane spanning region of the GABA receptor-chloride ionophore channel. Using the *Xenopus* oocyte expression system combined with site directed mutagenesis, it was clearly shown that the single amino acid substitution Ala³⁰² to Ser was indeed responsible for the insensitivity of the GABA receptor to dieldrin (ffrench-Constant *et al.*, 1993a). The same amino acid substitution has been shown to be responsible for the cyclodiene resistance in populations of *Ae. aegypti* (Thompson *et al.*, 1993), *M. domestica*, *T. castaneum*, and *Periplaneta americana*

(ffrench-Constant, 1994). Since all but *Ae. aegypti* and *M. domestica* are from different insect orders, the evolution of cyclodiene resistance appears to demonstrate an example of parallel evolution. Using the PCR-amplification of specific alleles (PASA) with PCR primers specific for the cyclodiene resistance gene, techniques have been developed for monitoring the frequency of this gene in populations of *Ae. aegypti* (ffrench-Constant *et al.*, 1994), *D. melanogaster* (Aronstein *et al.*, 1994; Aronstein *et al.*, 1995) and *T. castaneum* (Andreev *et al.*, 1994). PCR-based monitoring for cyclodiene resistance in *Drosophila* populations was compared directly with conventional insecticide bioassay and was found to have two advantages over the latter technique: (1) estimation of resistance frequency was more precise than that obtained by bioassays, and (2) estimations obtained by PCR required a smaller sample size (Aronstein *et al.*, 1994). However, monitoring using bioassays can not be abandoned because of the possibility of mutations in the GABA receptor gene other than that detected by PASA also giving rise to resistance. Given that resistance to insecticides by altered AChE (see section 1.4.3.1.) and altered Na⁺ channel protein (see section 1.4.3.3.) have also been shown to be due to point mutations (Pralavorio & Fournier, 1992; Amichot *et al.*, 1992), it may be possible to employ PASA to monitor the frequency of these genes in insect populations in the same way.

1.3.3.3. Altered sodium ion (Na⁺) channel

The target site of pyrethroids, the active components of pyrethrum (pyrethrins and cinerins), DDT and its analogues is the Na⁺ channel. The basis of this resistance

mechanism, also known as knock down resistance (*kdr*) or *super kdr* in houseflies (where a level of resistance has been reported above that seen with *kdr*), is a reduced affinity of the Na⁺ channel protein for the insecticide. The mechanism has been reported as being present in a number of strains of *M. domestica* (Ahn *et al.*, 1986a; Grubs *et al.*, 1988; Pauron *et al.*, 1989). A *kdr*-like mechanism of resistance has been reported in *D. melanogaster* (Jackson *et al.*, 1984; Ramaswami & Tanouye, 1989; Amichot *et al.*, 1992), *B. germanica* (Amichot *et al.*, 1992; Dong & Scott, 1991; Hemingway *et al.*, 1993; Dong & Scott, 1994; Scott & Dong, 1994), *Heliothis armigera* (Ahmad *et al.*, 1989), and the mosquitoes *C. quinquefasciatus* (Halliday & Georgiou, 1985; Amin & Hemingway, 1989) and *Ae. aegypti* (Amin & Hemingway, 1989; Hemingway *et al.*, 1989; Malcolm & Wood, 1982). In *M. domestica*, the *kdr* mechanism was found to be recessive to the susceptible state and was mapped to chromosome III (Ahn *et al.*, 1986b), a location corresponding to the Na⁺ channel gene (Williamson *et al.*, 1993). A partial cDNA of the gene was isolated (Williamson *et al.*, 1993) and, on sequencing, was found to have a 99% homology with a putative Na⁺ channel gene in *D. melanogaster* (Salkoff *et al.*, 1987). Restriction digest analysis of *M. domestica* having the 'susceptible' Na⁺ channel gene, the mutant *kdr*-type gene and the *super kdr*-type gene, using the partial cDNA of the *kdr* gene as a probe, revealed that the pattern of all three differed (the *super kdr* gene being found to have two distinct restriction digest patterns). This provided evidence that resistance has arisen through changes in the nucleotide sequence of the Na⁺ channel gene leading to changes in the amino acid sequence of the channel protein and a reduction in the affinity of the protein for pyrethroids. Further evidence of this came when the Na⁺ channel gene *sch*, located on chromosome II,

was cloned and sequenced from pyrethroid susceptible and resistant strains of *D. melanogaster*, a single nucleotide difference being found between the two resulting in a single amino acid substitution at position 1172 (Asp¹¹⁷² to Asn) (Amichot *et al.*, 1992). It was noted that, not only the Asn residue but a block of three residues Val-Asn-Asn (position 1170-1172) is fully conserved between this *Drosophila* sequence and all the vertebrate Na⁺ channel sequences reported to date (Amichot *et al.*, 1992). This is surprising as the region in which this block is located is on a region of the protein that is extracellular where one would expect such sequences to be much less conserved.

1.3.4. METABOLIC RESISTANCE

Metabolic resistance is the most commonly found of all the types of insecticide resistance mechanisms. This category of resistance includes qualitative and/or quantitative changes in enzymes which are then able to metabolise or sequester the insecticides before they can reach their target site. The products of this metabolism are generally more hydrophilic than the insecticide substrate and are hence more readily removed by excretion from the insect. There are three main enzyme groups involved in metabolic resistance: the glutathione *S*-transferases, the P⁴⁵⁰ monooxygenases and the esterases.

1.3.4.1. Glutathione *S*-transferases (GSTs)

Increased activity of GSTs can bring about resistance to DDT by

dehydrochlorination of this insecticide to DDE, and to OPs (phosphorothionates and their oxon analogues) by *O*-dealkylation (Hayes & Wolf, 1988; Lamoureux & Rusness, 1989). This mechanism has been found to be responsible for DDT and OP resistance in strains of *M. domestica* (Motoyama & Dauterman, 1978; Oppenoorth *et al.*, 1977; Clark & Shamaan, 1984; Ugaki *et al.*, 1985) and *An. subpictus* (Hemingway *et al.*, 1991), for DDT resistance in *Ae. aegypti* (Grant & Matsumura, 1989; Grant *et al.*, 1991), *An. sacharovi* (Hemingway *et al.*, 1985; Hemingway *et al.*, 1992) and *An. gambiae* (Prapanthadara *et al.*, 1993; Prapanthadara *et al.*, 1995), and for OP resistance in *D. melanogaster* (Morton, 1993) and the diamondback moth *Plutella xylostella* (Ku *et al.*, 1994). In addition, GSTs are possibly involved in insecticide resistance in the german cockroach *B. germanica* (Hemingway *et al.*, 1993). Multiple forms of GST have been found in *M. domestica* which, on the basis of immunological cross-reactivity, can be separated into two classes, GST-1 and GST-2 (Fournier *et al.*, 1992). Partial purification and characterization of GSTs from the DDT resistant Zands strain and susceptible G3 strain of *An. gambiae* also showed multiple forms of GSTs which, on the basis of interaction with chromatography materials and substrate kinetics, separated into at least two classes (Prapanthadara *et al.*, 1993). None of these GSTs showed cross-reactivity with antisera raised against *M. domestica* or *D. melanogaster* GST-1s demonstrating that there is considerable variation among the insect GSTs (Prapanthadara *et al.*, 1993). Similarly, no cross-reactivity was observed between an antiserum raised against purified GST-4 from *P. xylostella* and any of the GSTs in *M. domestica*, *D. melanogaster* or *Ae. aegypti* (Ku *et al.*, 1994). Of the seven GSTs partially purified from the DDT resistant Zands strain of *An. gambiae*, one GST (GST Va) possessed

60% of the total DDTase activity suggesting that it contributed most to DDT-metabolism in this strain (Prapanthadara *et al.*, 1995). The nucleotide sequence of a *M. domestica* GST-1 has a high degree of similarity to a GST-1 from *D. melanogaster* (Toung *et al.*, 1990) and the expressed proteins of the two GST-1s also have a high immunoreactivity. A DDT resistant strain of *M. domestica*, Cornell R, was found to have an increased level of mRNA coding for GST-1 and of the enzyme itself, but this was not accompanied by any gene amplification (Fournier *et al.*, 1992). This suggests, therefore, that the increase in GST-1 is brought about by an increase in gene transcription or mRNA stability. *In situ* hybridisation studies of GST genes in *D. melanogaster* have shown that the GST-1 and GST-2 gene families are situated at distinct loci on chromosome II (Beall *et al.*, 1992). Whilst the amino acid identity between different GST-1s is high (53-75%), there is a low identity between GST-1s and GST-2s (Beall *et al.*, 1992; Toung *et al.*, 1993). Genetic crossing experiments using a DDT resistant strain and a susceptible strain of *Ae. aegypti* suggest that GST-2 expression in the susceptible strain is under the control of a *trans*-acting transcriptional repressor (Grant & Hammock, 1992). It was suggested that in the DDT resistant strain of *Ae. aegypti* the transcriptional repressor is non-functional with the result that the GST-2 gene is over-expressed when compared to the susceptible strain. There is evidence of differential expression of GSTs involved in DDT resistance in larvae and adults of the Zands strain of *An. gambiae*. Larval selection of a sub-colony of this strain with DDT increased the resistance of larvae to this insecticide but not of emerging adults whilst selection of a subcolony at the adult stage resulted in increased resistance of adults but not larvae (Prapanthadara *et al.*, 1995).

1.3.4.2. P⁴⁵⁰ monooxygenases (P⁴⁵⁰s)

P⁴⁵⁰s catalyse the reduction of molecular oxygen by the incorporation of one oxygen atom into the substrate to give an oxidised product. The mechanism has been shown to be responsible for resistance to insecticides in a number of pest species of agricultural and public health importance (TABLE 1.2). Oxidative metabolism was also implicated as one of the mechanisms of resistance to the juvenoid methoprene in the R-methoprene strain of *M. domestica* (Hammock *et al.*, 1977). Using expression of the CYP6A1 P⁴⁵⁰ gene from an insecticide resistant strain of *M. domestica* in *E. coli*, together with a gene for NADPH-cytochrome P⁴⁵⁰ reductase from the same insect, Andersen *et al.* (1994) demonstrated that cyclodienes were metabolised by the CYP6A1 P⁴⁵⁰. The insecticide resistance-associated P⁴⁵⁰-B gene in *D. melanogaster* has been mapped to chromosome III (Waters & Nix, 1988). Analysis of the amount of P⁴⁵⁰ mRNA present in resistant and susceptible flies showed that more was present in the resistant strain (Waters *et al.*, 1992). This could either be due to an increase in transcription of the P⁴⁵⁰ gene and/or an increase in the stability of the mRNA. When the P⁴⁵⁰ genes from resistant and susceptible strains were compared, the gene from the resistant strain was found to be smaller in size (Waters *et al.*, 1992). On further analysis the susceptible gene was found to include a transposable element containing the sequence AUUUA not present in the resistant gene (Waters *et al.*, 1992). The presence of the sequence AUUUA has been shown to cause otherwise stable rabbit β -globin mRNA to become unstable leading to increased rates of degradation (Shaw & Kamen, 1986) and it was thought the absence of this sequence from the resistant P⁴⁵⁰ gene would

TABLE 1.2. Examples of pest species of public health importance reported as having a P⁴⁵⁰ monooxygenase-based mechanism of insecticide resistance.

Pest species	Groups of insecticides to which resistance shown	References
<i>Anopheles subpictus</i> (from Sri Lanka)	OPs	(Hemingway <i>et al.</i> , 1987)
<i>Culex quinquefasciatus</i>	OPs and pyrethroids	(Magnin <i>et al.</i> , 1988; Hemingway <i>et al.</i> , 1990; Khayrandish & Wood, 1993a)
<i>Blattella germanica</i>	OPs	(Siegfried <i>et al.</i> , 1990; Hemingway <i>et al.</i> , 1993)
<i>Musca domestica</i>	OPs and cyclodienes	(Welling <i>et al.</i> , 1974; Ugaki <i>et al.</i> , 1985; Takahashi & Yasutomi, 1987; Scott & Lee, 1993; Andersen <i>et al.</i> , 1994)
<i>Drosophila melanogaster</i>	OPs	(Waters & Nix, 1986; Waters & Nix, 1988)

bring about greater stability in the transcribed mRNA and hence account for the increase in amounts relative to that found in the susceptible strain. However, on analysing the genes from a large number of resistant and susceptible strains of *D. melanogaster*, the presence or absence of the transposable element was not found to be linked with resistance (Delpuech *et al.*, 1993).

Whilst the examples cited above show that the P^{450} monooxygenases can cause resistance through detoxifying insecticides, they can also have the reverse effect of increasing toxicity of insecticides to an organism. OPs are usually applied in the field in their thionate form, this form being of low toxicity to humans and having a high solubility in lipids which aids its entry into insects through the insects integument. Once inside the insect the P^{450} s convert the OP from its thionate form to its oxon analogue. The oxon analogues of OPs have a much higher affinity for their target site, AChE, than the thionates. For example, the anticholinesterase activity of malaoxon is 2000 times greater in *C. tarsalis* than malathion (Matsumura & Brown, 1961a). The role of P^{450} s in *M. domestica* microsomes in the conversion of thionate to oxon analog was investigated by Hatano and Scott (1993) using an antiserum raised against purified P^{450} . This study demonstrated that inhibition of P^{450} s prevented the activation of chlorpyrifos to chlorpyrifos-oxon. The use of piperonyl butoxide and other P^{450} inhibitors, employed in the laboratory to diagnose resistance caused by this mechanism and commercially in pyrethroid insecticide formulations to overcome P^{450} -based resistance, can actually increase resistance by preventing formation of the oxon analogues of OPs. In bioassays with both *An. stephensi* (Hemingway, 1982) and *C. quinquefasciatus* (Magnin *et al.*, 1988;

Khayrandish & Wood, 1993a; Bisset *et al.*, 1990), pre-exposure to piperonyl butoxide was shown to increase resistance to OPs.

1.3.3.3. Esterases

Insecticide resistance can be caused by qualitative and/or quantitative changes in esterases. Generally, esterases are involved with resistance to OPs and carbamates and, more rarely, with resistance to pyrethroids. As with other metabolic resistance mechanisms, both qualitative and quantitative-based esterase mechanisms prevent the insecticide from reaching its target site. Insect esterases involved with resistance may be of either A- or B-type [according to the classification of Aldridge (1953a; 1953b; 1993)] (see section 1.5.1.). Insect phosphoric triester hydrolases (phosphatases) fall into the former class, and carboxylesterases into the latter.

1.3.3.3.1. Phosphoric triester hydrolases (phosphatases) (EC 3.1.8)

Phosphatases detoxify OPs by cleaving off leaving groups resulting in the formation of non-toxic dialkyl phosphorothioic or phosphoric acids (Dauterman, 1971). It has been generally accepted that the properties of an OP that determine its susceptibility to cleavage are the same as those that enable it to inhibit AChE, ie. the electrophilic nature of the phosphorus atom (Wilkinson, 1971), and that consequently, phosphates are more readily hydrolysed by phosphatases than phosphorothioates. When phosphatase activity was measured in diazinon and parathion resistant and susceptible strains of the house fly *M. domestica* low levels

of activity were detected in the resistant strains whilst in the susceptible strains, no activity could be detected (Oppenoorth & Van Asperen, 1960; Oppenoorth & Van Asperen, 1961; Welling *et al.*, 1970). The product formed from the metabolism of paraoxon was shown to be diethyl phosphate (Welling *et al.*, 1970). Using the esterase inhibitor *n*-propyl paraoxon the metabolism of paraoxon was inhibited, indicating that metabolism was due to phosphatase hydrolysis (Oppenoorth & Van Asperen, 1961; Welling *et al.*, 1970). It was claimed by Matsumura and Hogendijk (1964) that the cleavage of malathion (a phosphorothionate) could be detected *in vitro*, the dialkyl phosphorothioic acid being detected. However, no such degradation of malathion could be detected by Welling *et al.* (1970) when attempting to confirm this finding. There are reports of phosphatase metabolism of paraoxon in a parathion resistant strain of aphid *Myzus persicae* and of parathion and diazinon in the cockroach *Periplaneta americana* (although this was not associated with resistance), both studies being carried out *in vitro* on partially purified preparation of esterases (Oppenoorth & Voerman, 1975; Matsumura & Sakai, 1968). In addition, phosphatase activity has been shown to be responsible for methyl parathion resistance in the tobacco budworm *Heliothis virescens* (Konno *et al.*, 1990).

1.3.3.3.2. Carboxylesterases

Qualitative alterations in esterases cause resistance to insecticides through an increase in affinity and/or in the rate of hydrolysis. Alternatively, resistance may arise through a quantitative change in esterases that are already able to hydrolyse insecticides, but are not present in sufficient quantities in wild-type, susceptible

insects to cause resistance. Despite the wide variety of OPs used against insects in the field, most of the reported cases of resistance to OPs through carboxylesterases have been for malathion. The reason for this is the susceptibility of the carboxylester groups of malathion to hydrolysis, the products being malathion mono- and dicarboxylic acids which are water soluble, nontoxic and easily eliminated by excretion. An increase in the amount of mono- and dicarboxylic acids in resistant versus susceptible strains due to increased malathion carboxylesterase-based metabolism occurs in the mosquitoes *An. culicifacies* (Malcolm & Boddington, 1989) and *An. stephensi* (Hemingway, 1982), the house fly *M. domestica* (Matsumura & Hogendijk, 1964) and the sheep blowfly *Lucilia cuprina* (Whyard & Walker, 1994). Cross-resistance between malathion and phenthoate has been reported for strains of the mosquitoes *C. tarsalis* and *An. stephensi*, again with carboxylesterases acting on the insecticide's carboxylester bond (Georghiou, 1969; Hemingway, 1982). However, carboxylesterases have also been found to be responsible for resistance to OPs that do not have carboxylester bonds, in these cases the enzymes acting on the phosphate ester (P-O) bonds of the insecticide. Carboxylesterase hydrolysis in a strain of *L. cuprina* caused resistance to diazinon (Hughes & Raftos, 1985), and the same mechanism was found to cause resistance to parathion and methyl-parathion in *C. tarsalis* (Apperson & Georghiou, 1975; Prabhaker *et al.*, 1987) and resistance to fenitrothion in the small brown planthopper *Laodelphax striatellus* (Sakata & Miyata, 1994). In addition to the evidence of the role of carboxylesterase hydrolysis in OP resistance, evidence has also come from the use of carboxylesterase inhibitors. Pre-exposure of OP resistant *An. arabiensis* to the carboxylesterase synergist triphenyl phosphate (TPP) (20%) for one hour followed by exposure to

5% malathion gave almost 100% mortality whereas exposure to 10% monooxygenase/^{synergist} piperonyl butoxide for one hour followed by exposure to 5% malathion for four hours gave mortalities of 10-12%, showing that resistance to malathion in this strain was mainly due to the carboxylesterase-based mechanism (Hemingway, 1983). Similarly, the anti-carboxylesterase synergists DEF (S,S,S-tributyl phosphorothionate), IBP (S-benzyl O,O-diisopropyl phosphorothionate), DFP (diisopropylfluorophosphate) and TPP have been used to substantiate the role of carboxylesterase hydrolysis in OP resistance in strains of *C. tarsalis*, *An. stephensi*, *Tetranychus urticae*, *M. domestica* and *L. cuprina* (Apperson & Georghiou, 1975; Matsumura & Voss, 1965; Matsumura & Hogendijk, 1964; Whyard & Walker, 1994). Malathion hydrolysis by a *M. domestica* malathion carboxylesterase was inhibited by the esterase substrates α - and β -naphthyl acetate (NA) (Picollo de Villar *et al.*, 1983). The changes in the conformation of carboxylesterases that enable them to hydrolyse OPs have been observed to have a varying effect on hydrolysis of the substrates α - and β -NA. In the mosquitoes *An. arabiensis*, *An. culicifacies* and *An. stephensi* there was no difference between the levels of activity with these substrates in the susceptible and OP-resistant strains (Hemingway, 1982; Hemingway & Georghiou, 1984; Malcolm & Boddington, 1989). These results were interpreted as showing that there was no quantitative change in the amount of carboxylesterase present in the resistant strain as compared to the susceptible. In the two-spotted spider mite *T. urticae*, activity with β -NA was lower in a resistant strain when compared with a susceptible strain (Matsumura & Voss, 1965), and in the sheep blowfly *L. cuprina* the E₃ esterase in a susceptible strain hydrolysed both α - and β -NA whilst the mutant E₃ esterase in a resistant strain could hydrolyse

neither of these esterase substrates (Hughes & Raftos, 1985). In some OP and carbamate resistant strains of *M. domestica*, reduced activity was seen with the substrate methyl *n*-butyrate compared to a susceptible strain (Oppenoorth & Van Asperen, 1960; Oppenoorth & Van Asperen, 1961; Plapp & Bigley, 1961). All these examples suggest a qualitative change in the carboxylesterases in the resistant strain that made them more efficient at hydrolysing OPs but less efficient at hydrolysing α - and β -NA. In both the house fly *M. domestica* and the sheep blowfly *L. cuprina*, carboxylesterases in resistant strains showed increased levels of activity with both α - and β -NA, and malathion (Picollo de Villar *et al.*, 1983; Whyard & Walker, 1994) but, in contrast to observations with *L. striatellus* (Sakata & Miyata, 1994), this was accompanied by increases in the K_m (in both species of insect) and V_{max} (in *L. cuprina*) suggesting that resistance in these cases is due to a qualitative change in carboxylesterase in the resistant strains leading to increased hydrolysis of malathion. In the mosquito *C. tarsalis* using α - and β -NA, and in the small brown planthopper *L. striatellus* using α -NA, an increased level of activity was observed in resistant strains when compared to a susceptible (Georghiou & Pasteur, 1978; Sakata & Miyata, 1994; Sakata & Miyata, 1994). No differences were found in the kinetic constant K_m or V_{max} , or the specific activity to α -NA between the carboxylesterases in the resistant and susceptible strains of *L. striatellus* (Sakata & Miyata, 1994), suggesting that in this case, and in the case of OP resistance in *C. tarsalis*, resistance is due to quantitative changes in carboxylesterases leading to increased levels of malathion hydrolysis.

Esterases have been implicated in the metabolism and detoxification of some

pyrethroids in vertebrates, both by the metabolites produced from pyrethroids tested, and the inhibition of metabolism by use of the esterase synergist DEF (also an anti-monooxygenase synergist) and of esterase inhibitors (OPs and carbamates) (Abernathy & Casida, 1973; Abernathy *et al.*, 1973; Suzuki & Miyamoto, 1978). Both the porina moth *Wiseana cervinata* and the whitefly *B. tabaci* have esterases which give tolerance to pyrethroids (Chang & Jordan, 1983; Ishaaya *et al.*, 1987), the wild-type insects having esterases which are pre-adapted to the metabolism of these insecticides. Again, pyrethroid metabolism was largely inhibited by exposure to OPs and carbamates, thus providing evidence that esterase hydrolysis is responsible for the observed tolerance. The milkweed bug *Oncopeltus fasciatus*, the cockroach *B. germanica*, the house fly *M. domestica*, the cabbage looper *Trichoplusia ni* and the yellow mealworm *Tenebrio molitor* were all found to have esterases capable of hydrolysing resmethrin and tetramethrin, but not S-bioallethrin, hydrolysis being inhibited by 1-naphthyl N-propylcarbamate and DEF (Jao & Casida, 1974). It should be pointed out that hydrolysis of pyrethroids in the strains tested did not constitute tolerance or resistance. In the cattle tick *B. microplus*, pyrethroid hydrolysing esterases were found to be responsible for resistance (de Jersey *et al.*, 1985). Whilst the esterase activity in the resistant and susceptible strains, using the substrate *p*-nitrophenyl butyrate, were the same, small differences in substrate specificity and differences in the banding pattern after isoelectric focusing were detected. This suggests that a qualitative change in esterases leading to increased hydrolysis rates with pyrethroids was responsible for resistance. However, in a decamethrin-resistant strain of the Egyptian cotton leafworm *Spodoptera littoralis*, increases in the hydrolysis of the esterase substrates α - and β -

NA, and *p*-nitrophenyl acetate suggest a quantitative change in esterases. The toxicity of pyrethroids was increased in the resistant strain by pre-exposure to DEF providing evidence that esterases contribute to the resistance to pyrethroids in this species. In a Guatemalan strain of *An. albimanus* biochemical assays and bioassays revealed that esterase hydrolysis caused cross-resistance to fenitrothion and deltamethrin, resistance to both insecticides being nearly abolished by DEF (Brogdon & Barber, 1990). An increase in the level of activity with α -NA both in biochemical assays and on native polyacrylamide gels suggested that there has been a quantitative change in an esterase in the resistant strain.

A correlation between insecticide resistance and increases in carboxylesterase activity have been detected, both by biochemical assays and staining of native polyacrylamide gels, using general esterase substrates. As can be seen above, this increase in activity may be associated with qualitative changes in esterases which has led to an increase in the rate of hydrolysis of both esterase substrates and insecticides, or by a quantitative change in esterases that are already able to hydrolyse both substrates and insecticides. A third way in which esterases can bring about resistance to insecticides is by an increase in the amounts of non-specific esterase present in an insect that are then able to sequester insecticides. These esterases rapidly bind the insecticides followed by a much slower turnover step, to prevent the insecticides from reaching their target site. This mechanism has been implicated in OP resistance and/or pyrethroid resistance in a number of pest species of public health and agricultural importance including several species of mosquitoes (TABLE 1.3). Whilst both the elevated esterases in *C. quinquefasciatus*

TABLE 1.3. Examples of pest species of public health and agricultural importance reported as having an elevated esterase-based mechanism of insecticide resistance.

Pest species	Groups of insecticides to which resistance shown	References
<i>Culex quinquefasciatus</i>	OPs	(Georghiou & Pasteur, 1978; Hemingway & Georghiou, 1984; Peiris & Hemingway, 1990b; Wirth <i>et al.</i> , 1990)
<i>Culex pipiens</i>	OPs	(Pasteur <i>et al.</i> , 1981a; Pasteur <i>et al.</i> , 1981b; Villani <i>et al.</i> , 1983; Villani & Hemingway, 1987; Maruyama <i>et al.</i> , 1984; Fournier <i>et al.</i> , 1987)
<i>Culex tritaeniorhynchus</i>	OPs	(Kamimura & Maruyama, 1983; Takahashi & Yasutomi, 1987)
<i>Aedes aegypti</i>	OPs	(Field <i>et al.</i> , 1984; Mourya <i>et al.</i> , 1993)
The tobacco aphid <i>Myzus nicotianae</i>	OPs	(Abdel-Aal <i>et al.</i> , 1990; Abdel-Aal <i>et al.</i> , 1992)
The peach-potato aphid <i>Myzus persicae</i>	OPs and pyrethroids	(Needham & Sawicki, 1971; Needham & Devonshire, 1975; Beranek, 1974; Devonshire & Moores, 1989; Devonshire, 1977; Abdel-Aal <i>et al.</i> , 1990)
The tobacco whitefly <i>Benisia tabaci</i>	OPs	(Byrne & Devonshire, 1991)
The rice brown planthopper <i>Nilaparvata lugens</i>	OPs	(Chen & Sun, 1994; Hemingway <i>et al.</i> , 1995; Karunaratne & Hemingway, 1995)
The green rice leafhopper <i>Nephotettix cincticeps</i>	OPs	(Motoyama <i>et al.</i> , 1984)
The greenbug <i>Schizaphis graminum</i>	OPs	(Ono <i>et al.</i> , 1994)
The german cockroach <i>Blattella germanica</i>	OPs	(Hemingway <i>et al.</i> , 1993; Prabhakaran & Kamble, 1995; Scharf <i>et al.</i> , 1995)
The Colorado beetle <i>Leptinotarsa decemlineata</i>	Pyrethroids	(Lee & Clark, 1994)

and the elevated E4 esterase in *Myzus persicae* were shown to have a very high affinity for OPs (as measured by their bimolecular rate constants, k_a s) (Karunaratne *et al.*, 1993; Small *et al.*, 1995; Devonshire, 1977), esterase E4 hydrolysed OPs at a faster rate than the *C. quinquefasciatus* esterases, showing that the role of esterase E4 is one of hydrolysis as well as sequestration whereas that of the *C. quinquefasciatus* esterases is solely one of sequestration. In addition, the aphid esterase E4, which is kinetically identical to the equivalent esterase in susceptible strains (Devonshire, 1977), was shown to hydrolyse *trans*-permethrin and to bind reversibly with deltamethrin and kadethrin (Devonshire & Moores, 1989) so that variants having elevated levels of esterase E4 were resistant to these pyrethroids. As with the esterase-based mechanisms mentioned above, the correlation of the elevated-esterase based mechanism with OP resistance was substantiated by use of the synergists DEF, IBP and TPP in bioassays of various OP resistant strain of *C. quinquefasciatus* (Georghiou & Pasteur, 1978; Hemingway & Georghiou, 1984; Bisset *et al.*, 1990; Wirth *et al.*, 1990). Interesting results were obtained by Ranasinghe & Georghiou (1976) after selecting an OP-resistant strain of *C. quinquefasciatus* for nine generation with three regimens; temephos only, temephos plus DEF and temephos plus PB. Selection with temephos only increased resistance to this insecticide and selection with temephos plus PB increased resistance, but to a lesser extent. These results are not unexpected, the temephos plus PB selected subcolony being under less selection pressure than the temephos selected subcolony because of the prevention of monooxygenase metabolism of temephos to its more toxic oxon analogue. However, selection with temephos plus DEF decreased resistance to a near-normal level of susceptibility. Plapp (1986) suggested that this

result may have been due to a selection of an esterase that increased its ability to recognise and bind DEF and simultaneously lost its ability to recognise and bind temephos. However, this explanation fails to recognise the non-specific nature of the elevated *Culex* esterases. Whilst exposure to temephos plus DEF may conceivably have selected for forms of the elevated esterase that were better able to bind DEF, this would not have completely removed the ability of the esterase to bind temephos. It is known that the frequency of the elevated esterase-based mechanism selected by malathion spraying of populations in Cuba declined once spraying of malathion had ceased (Rodriguez *et al.*, 1993) suggesting that, in the absence of a selective advantage of the mechanism, its possession actually puts individuals at a selective disadvantage in relation to those that do not possess it. An alternative explanation might, therefore, be that the inclusion of DEF in the temephos selection negated the advantage of possessing the elevated esterase-based mechanism and that individuals possessing these esterases were selected out by other selection pressures.

1.4. ESTERASES

1.4.1. CLASSIFICATION

The term 'esterase' covers a wide range of enzymes that are able to catalyse the hydrolysis of carboxylic and phosphoric acid esters. By using the variable sensitivity of esterases to inhibition by paraoxon, Aldridge (1953a; 1953b; 1993) classified esterases into two main groups; those esterases unaffected by the presence

of paraoxon are termed A esterases and those inhibited by paraoxon in a progressive and time-dependent reaction are termed B esterases. The A esterases hydrolyse OPs (possibly through the acylation of an active site cysteine) (Aldridge, 1993), require metal ions for their activity and are sensitive to inhibition by metal ions other than those required for activity. Esterases coming within this grouping are now termed 'phosphoric triester hydrolases' and, under the nomenclature of the International Union of Biochemistry (IUB) are numbered EC 3.1.8. (Reiner, 1993; Walker, 1993). The B esterases have been shown to have a serine residue in the active site, hence the alternative name 'serine hydrolases', and it is the acylation of this that causes inhibition of these enzymes. Esterases coming within this grouping are now mainly termed 'carboxylesterases' and under IUB nomenclature are numbered EC 3.1.1.1 (Reiner, 1993; Walker, 1993).

Various other nomenclatures have been adopted to classify esterases within a species or group of species. Early classifications of vertebrate esterases used their varying substrate specificities. However, many of these esterases are non-specific with regard to the substrates. Therefore, this type of classification has been abandoned. Mentlein *et al.* (1984; 1985a) proposed a nomenclature of rat liver microsome esterases that used the most prominent natural substrates of these esterases. These esterases were termed monacylglycerol lipase, palmitoyl-CoA hydrolase, palmitoyl-carnitine hydrolase etc. Gaustad *et al.* (1992) adopted an alternative classification for rat liver esterases based on their isoelectric-point (pI). The isolated esterases were named 6.4, 6.2, 6.0, 5.2 and 5.0. However, ^{the} characteristic most widely used for classification has been the relative mobility of esterases on

native gels. For example, mouse and rat esterases (Peters, 1982; Simons & Vander Jagt, 1977), and german cockroach esterases and sheep blowfly esterases (Prabhakaran & Kamble, 1993; Parker *et al.*, 1991) have all been classified according to this system. Yet another system has been used for *D. melanogaster* esterases, these esterases being classified by combining their relative mobility on both native gels and isoelectric focusing gels (Healy *et al.*, 1991).

In the *C. pipiens* complex of mosquitoes, esterases involved with OP resistance have been classified previously according to their relative mobility on native gels (starch and polyacrylamide) and their preferential hydrolysis of the substrates α - and β -naphthyl acetate (NA) (Georghiou & Pasteur, 1978; Raymond *et al.*, 1987). Those esterases preferentially hydrolysing α -NA were termed esterases A and those preferentially hydrolysing β -NA were termed esterases B. Subscripts 1, 2 etc. were used after the letter to denote the order in which the esterases were discovered. Three major esterase phenotypes have been reported for populations of the *C. pipiens* complex: A₁ in Southern France and Italy (Pasteur *et al.*, 1981a; Severini *et al.*, 1993); B₁ in populations from California, Cuba, Central America and Asia (Georghiou & Pasteur, 1978; Bisset *et al.*, 1990; Georghiou, 1992); and esterases A₂ and B₂ which is found worldwide (Villani *et al.*, 1983; Raymond *et al.*, 1987; Hemingway *et al.*, 1990; Wirth *et al.*, 1990; Peiris & Hemingway, 1993; Rivet *et al.*, 1994). This system has largely broken down as esterases which have the same electrophoretic mobilities but different DNA restriction digest patterns have been given different names (Poirie *et al.*, 1992). In addition, this system invites confusion with that of Aldridge (1953a; 1953b) which also uses the letters A and B. A revised

nomenclature was, therefore, recommended by Karunaratne (1994) and adopted by Vaughan & Hemingway (1995) in which the letters A and B are replaced by the greek letters α and β , with numerical superscripts denoting sequence variants with the same electrophoretic mobility. Hence the two amplified esterase B₁s in the Californian TEM-R and Cuban MRES strains which have been shown to differ in their inferred amino acid sequence but are electrophoretically identical (Mouches *et al.*, 1990; Vaughan *et al.*, 1995) are now Est β 1¹ and Est β 1² respectively, and the non-elevated esterase B from the Sri Lankan PelSS strain, again with the same electrophoretic mobility but varying in amino acid sequence (Karunaratne, 1994; Vaughan *et al.*, 1995), becomes Est β 1³. The elevated esterases in the OP-resistant Sri Lankan strain PelRR, of which the sequences have recently been published (Vaughan *et al.*, 1995; Vaughan & Hemingway, 1995) are now termed Est α 2¹ and Est β 2¹, having been termed A₂ and B₂ under the previous nomenclature. The genes coding for these esterases are written in lower case and in italics. Hence, the gene coding for esterase Est α 2¹ is *est* α 2¹ and that coding for Est β 2¹ is *est* β 2¹.

1.4.2. PHYSICOCHEMICAL PROPERTIES

1.4.2.1. Physical properties

The majority of vertebrate carboxylesterases are monomers and have a molecular weight of about 60 kDa, although a few forms have been reported as being stable as trimers (Heymann, 1980). For example, the native molecular weights of several mouse esterases, the genes of which are all located on chromosome 8, were 45-55

kDa (Peters, 1982), that of the esterase ES-1 on the same chromosome was 65 kDa (Kadner *et al.*, 1992), and that of a rat liver microsomal esterase was 59 kDa (Alexson *et al.*, 1993). However, Diemling & Gaa (1992) reported that the mouse esterase ES-29 had a native molecular weight of 130 kDa, indicating that this esterase is a dimer in its active form, and an esterase purified from rat lung was shown to be a trimer with monomeric units of 60 kDa (Gaustad *et al.*, 1991). Human monocyte-specific esterase is a trimer with 63.4 kDa monomeric units and a human alveolar macrophage esterase is also a trimer with 60 kDa subunits (Munger *et al.*, 1991). The majority of insect carboxylesterases are also monomers and have molecular weights within the range of those reported for vertebrate carboxylesterases although there are several exceptions to this (TABLE 1.4). It is possible with *C. pipiens* Est α 1 (Fournier *et al.*, 1987) that the formation of the dimer was a temporary association caused by high enzyme concentrations.

The isoelectric points of B esterases are usually in the range pH 4.7-6.5 (Heymann, 1980). Two purified human liver carboxylesterases were reported to have pI values of 5.2-5.8 and mouse, rat and rabbit liver carboxylesterases had pI values of 4-7 (Kao *et al.*, 1985a; Simon *et al.*, 1985; Mentlein *et al.*, 1984; Mentlein *et al.*, 1985a; Mentlein *et al.*, 1985b). Two esterases from an insecticide resistant strain of *M. domestica* were reported to have pIs of 5.1 and 5.3 (Kao *et al.*, 1985b), whilst the *N. lugens* esterases E₁, E₂ and E₃ had pI values of 4.7-4.9 (Chen & Sun, 1994), the *B. germanica* esterases E5, E6 and E7 had pI values of 4.7-5.0 (Prabhakaran & Kamble, 1995), twenty-two soluble *D. melanogaster* esterases had pI values of 3.8-6.2 (Healy *et al.*, 1991), and the *L. decemlineata* esterases had pI values of 4.5-4.8 (Lee

TABLE 1.4. The native molecular weights and native forms of insect esterases.

Esterases	Native molecular weight	Native form	References
<i>Myzus persicae</i> esterases E4 and FE4	65 and 66 kDa	Monomers	(Devonshire <i>et al.</i> , 1986b)
<i>Drosophila melanogaster</i> esterase-C	65 kDa	Monomer	(Holwerda & Morton, 1983)
<i>Nilaparvata lugens</i> esterases E ₁ , E ₂ and E ₃	62-64 kDa	Monomers	(Chen & Sun, 1994)
<i>Blattella germanica</i> esterases E5, E6 and E7	53-57 kDa	Monomers	(Prabhakaran & Kamble, 1995)
<i>Leptinotarsa decemlineata</i> esterase pl 4.8, esterase pl 4.5	49 kDa, 60 kDa and 33 kDa	Monomer, Heterodimer	(Lee & Clark, 1994) (Lee & Clark, 1994)
<i>Musca domestica</i> esterases	220 kDa	Not determined	(Kao <i>et al.</i> , 1985b)
<i>Culex quinquefasciatus</i> Estα2 ¹ , Estβ1 ¹ and Estβ2 ¹	67, 67 and 60 kDa	Monomers	(Fournier <i>et al.</i> , 1987; Ketterman <i>et al.</i> , 1992; Jayawardena, 1992)
<i>Culex pipiens</i> Estα1	118-134 kDa	Homodimer	(Fournier <i>et al.</i> , 1987)
<i>Culex tarsalis</i> esterase	16 kDa	Monomer	(Matsumura & Brown, 1963)

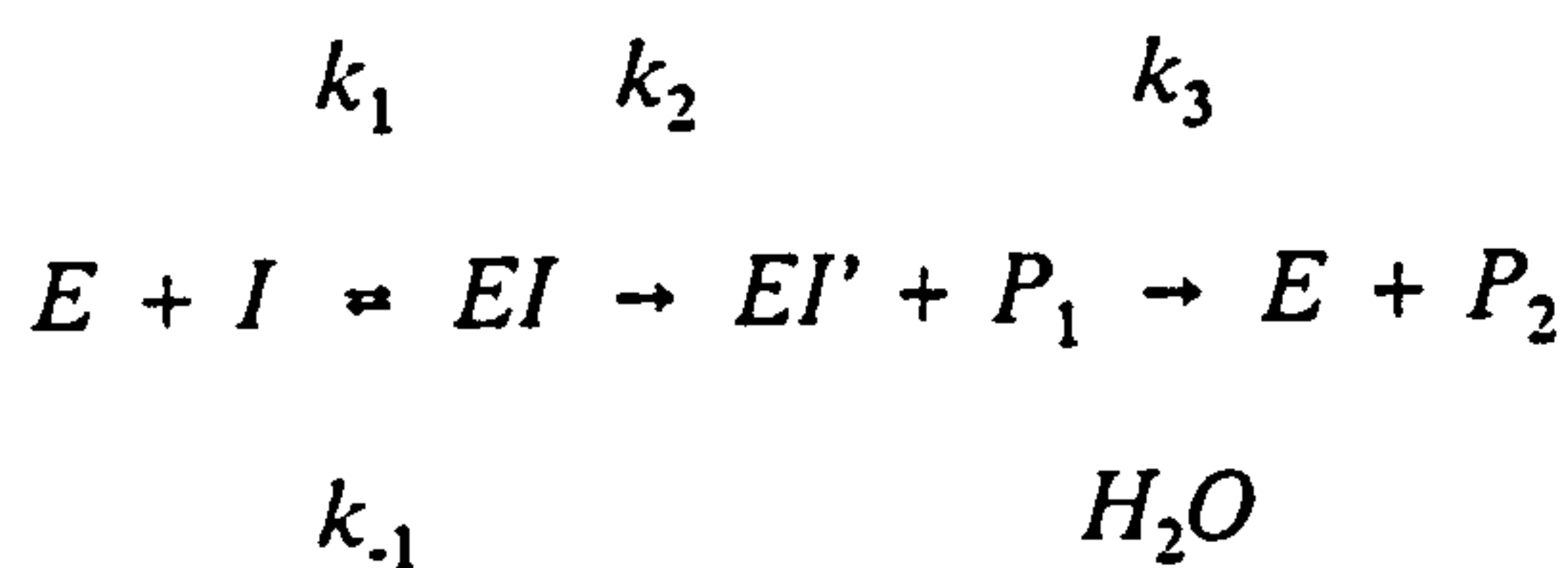
& Clark, 1994). The esterases Est α 1, Est β 1¹, Est α 2¹ and Est β 2¹ from mosquitoes of the *C. pipiens* complex had pIs in the range 5-6 (Fournier *et al.*, 1987; Kettermann *et al.*, 1992; Jayawardena, 1992).

Glycosylation is an important characteristic of proteins having a marked influence on their biological functions. The steric interaction of peptides and attached oligosaccharides influence polypeptide-folding events that lead to the tertiary structure of proteins. The attachment of particular oligosaccharides to a newly synthesised protein, can also make that protein a target for transport to a particular intracellular organelle, or for export (by secretion) or placement on the outer surface of cells. The question of whether esterases are or are not glycosylated is, therefore, not only a useful characteristic for classification, but may also provide information on how glycosylation effects the function of esterases and provides clues as to where they might be localised. Mammalian carboxylesterases are oftenglycosylated. For example, ES-1 from the mouse is known to be a glycoprotein (Kadner *et al.*, 1992), as are the human monocyte-specific esterase (Munger *et al.*, 1991) and all the microsomal rat liver carboxylesterases (Robbi & Beaufay, 1986; Robbi & Beaufay, 1988). The glycosylated nature of the *Myzus persicae* esterase E4 has been shown both by its high affinity for lectin (in affinity chromatography using Con-A Sepharose) and by native electrophoresis followed by staining for the presence of sugars. It has been shown that oligosaccharides attached to *Myzus persicae* esterases E4 and FE4 may account for 6-8 kDa of its total molecular weight, as determined by both *in vitro* translation of mRNA (Devonshire *et al.*, 1986b) and analysis of deduced amino acid sequences (Field *et al.*, 1993). The latter

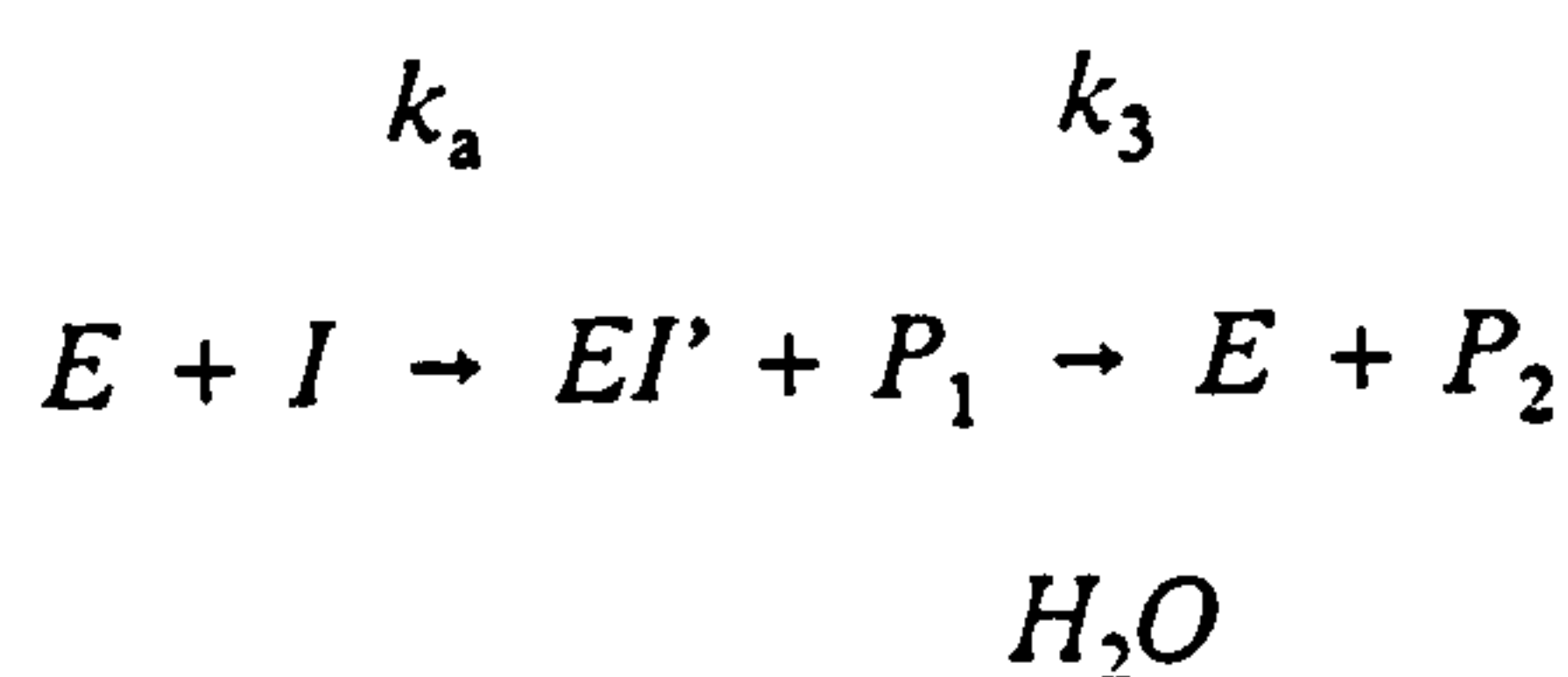
study showed that esterases E4 and FE4 both had 5 possible glycosylation sites. The *C. quinquefasciatus* esterases Est α 2¹ and Est β 2¹ did not interact with Con-A Sepharose suggesting that they are not glycosylated (Karunaratne, 1994). Analysis of the deduced amino acid sequence of the cDNA of the Est α 2¹ esterase showed only two possible glycosylation sites, neither of which was conserved in other esterases and which were situated only 4 and 31 amino acids from the putative active site serine residue (Vaughan & Hemingway, 1995). Neither of the cDNA sequences of these *C. quinquefasciatus* esterases were found to contain hydrophobic signal sequences suggesting that they are not exported out of the cell (Vaughan & Hemingway, 1995). The multiple forms of carboxylesterase from *L. decemlineata* were all determined, by the concanavalin-A staining method, to be glycoproteins (Lee & Clark, 1994).

1.4.2.2. Chemical properties

Carboxylesterases are able to detoxify insecticides by catalysing the hydrolysis of carboxylester, carboxyamide ester and carboxythioesters (Ahmad & Forgash, 1976) and also certain phosphoric acid esters. The majority of carboxylesterases contain a serine-histidine-glutamate catalytic triad, with a few having an aspartic acid residue in place of the glutamine (Cygler *et al.*, 1993). The inhibition of B esterases by OPs and carbamates occurs by their rapid esterification of the active site serine. This is usually followed by a much slower hydrolysis of the insecticide resulting in the reactivation of the enzyme. The generally accepted reaction mechanism of enzyme with carbamates or organophosphates is;

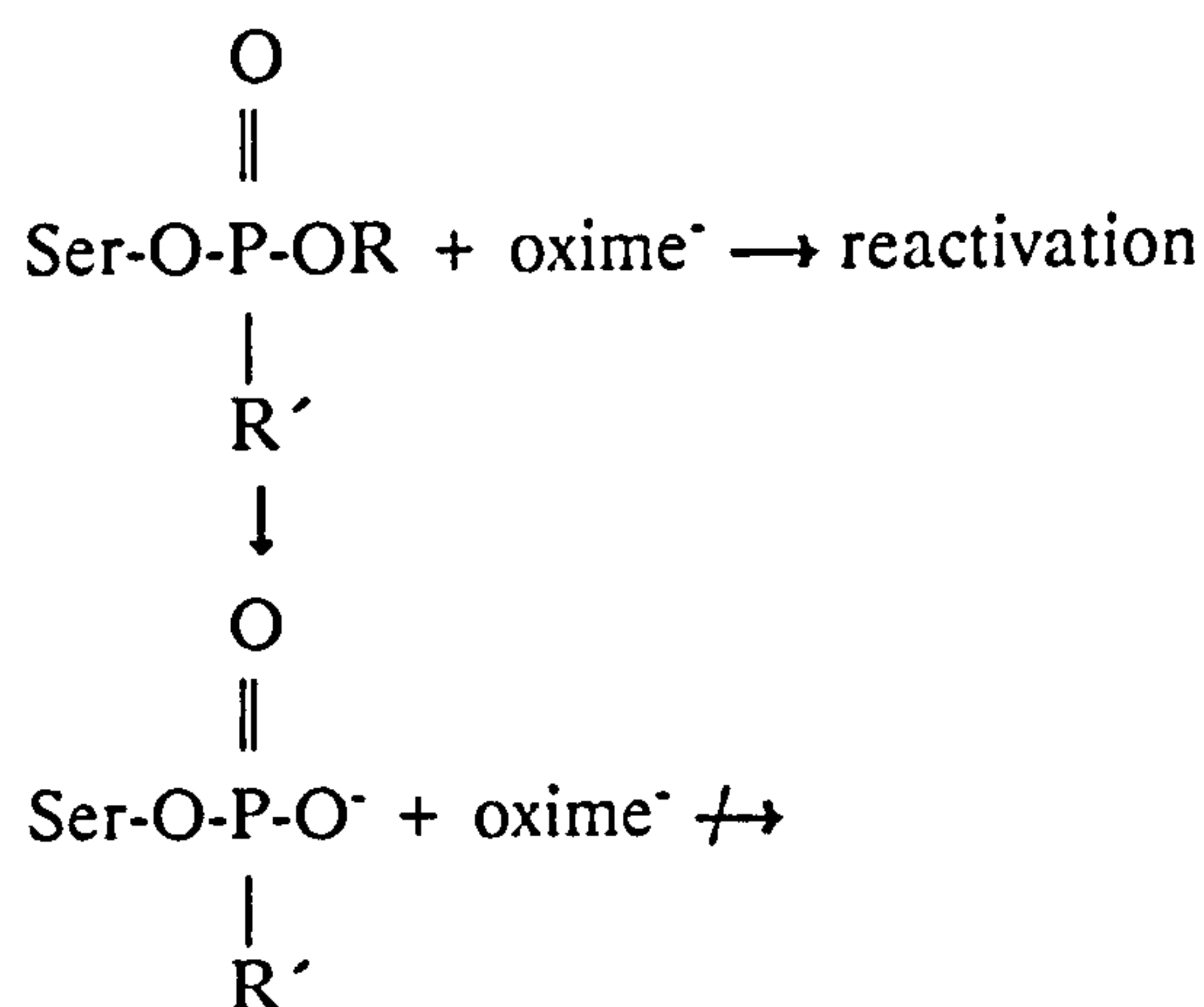


or, where the concentration of the Michaelis complex is very small because of the rapid acylation of the enzyme;



where E is the enzyme, I is the inhibitor, EI is the Michaelis complex and EI' is the acylated enzyme, P_1 is the first product (an alcohol) and P_2 is the second product (an acid). It can be seen from these equations that the reactions of inhibition are strictly analogous to those for substrate hydrolysis and that the insecticides are merely poor substrates for these esterases. However, in contrast to the inhibition of esterases with OPs and carbamates which is reversible, with cholinesterase inhibition by phosphonates and phosphoroamidates and some phosphates, following the formation of the acylated enzyme complex, the feasibility of reactivation decreases in a time-dependent manner, even with known reactivating agents such

as fluoride salts and oximes ie. inhibition is irreversible. Under these circumstances the enzyme is said to have 'aged' (Johnson, 1987). The ageing reaction involves the cleavage of an R-O-P (with phosphates and phosphonates) or R-NH-P bond (with phosphoramidates) and the generation of a negatively charged substituent on the enzyme which is bound stably. The dealkylation of an OP and its effect on oxime reactivation can be written as:



Where Ser-O-P is the covalent bond formed by the phosphorylation of Ser-OH, the active site serine. The mechanism for the ageing process is generally believed to be a secondary transphosphorylation reaction. Upon inhibition, the acyl group initially becomes attached to the imidazole of the active site histidine where it is susceptible to reactivation by oximes in analogy to the labile binding of the acyl-enzyme intermediate. Thereafter, in a secondary reaction, a gradual transphosphorylation takes place from the histidine to the serine and it is in this position that the acylated enzyme complex becomes stable (Berends, 1987). It is not clear whether substrates go through the same acylated esterase intermediate step because the intermediate has yet to be isolated (Aldridge, 1993). For inhibitors, the acylated

enzyme is formed very quickly ie the k_a is very high, but it is either stable (as mentioned above) or its rate of hydrolysis is very slow and becomes the rate limiting step. It is this high affinity for OPs and carbamates when combined with increased amounts of esterases, that make them such an effective resistance mechanism.

Estimates of the kinetic constants k_a (the bimolecular rate constant) and k_3 (the reactivation rate constant) have shown that the role of the *C. quinquefasciatus* esterases Est α 2 and Est β 2 in OP resistance is one of sequestration (Kettermann *et al.*, 1992; Hemingway *et al.*, 1993; Karunaratne *et al.*, 1993; Kettermann *et al.*, 1993), whilst the role of the *Myzus persicae* esterase E4 is both by sequestration and hydrolysis (Devonshire, 1977; Devonshire & Moores, 1982). A correlation was found between k_a and cross-resistance to OPs, the higher the affinity for the OP (its oxon analogue), the higher the cross-resistance to it (Hemingway *et al.*, 1993; Karunaratne *et al.*, 1993). The aphid esterase E4, which is kinetically identical to the equivalent esterase in susceptible strains (Devonshire, 1977), was shown to hydrolyse *trans*-permethrin and to bind reversibly with deltamethrin and kadethrin (Devonshire & Moores, 1989) so that variants having elevated levels of esterase E4 were resistant to these pyrethroids.

The exact physiological role of most of the non-specific esterases is difficult to determine. However, their localisation (see section 1.4.3.) in both vertebrates and insects suggests that their function is to hydrolyse xenobiotics. The rat liver microsomal carboxylesterases have been shown to hydrolyse a large number of physiological and xenobiotic substrates (Mentlein *et al.*, 1980; Mentlein *et al.*, 1984;

Mentlein *et al.*, 1985a; Mentlein *et al.*, 1985b). Kettermann *et al.* (1992) used a variety of substituted glycerol substrates to examine the possible physiological role of the *C. quinquefasciatus* esterase Est α 2¹. This esterase appeared to have a preference for medium-chain-length mono- and diacylglycerols and it was suggested that this was evidence to support the involvement of carboxylesterases in lipid-fatty acid metabolism in addition to their insecticide detoxification role.

The kinetics of AChE is perhaps the best defined of all the carboxylesterases. The catalytic mechanism is one of the most efficient known, enabling it to turnover 25,000 acetylcholine molecules per second (Bazelyansky *et al.*, 1986). Indeed, the rate of hydrolysis of this substrate is diffusion limited. Catalysis is thought to involve a charge relay system, an electron being transferred within the active site triad. Determination of the three-dimensional structure of the AChE in *Torpedo californica* revealed that a negatively charged glutamate residue is able to draw a hydrogen atom from an adjacent histidine residue which, in turn, draws a hydrogen atom from the final residue in the triad, serine (Sussman *et al.*, 1991). This charge relay system allows nucleophilic attack on acetylcholine resulting in the acylated enzyme. The covalent enzyme-acetylcholine bond is then hydrolysed resulting in the release of acetate. The catalytic site of AChE is contained within the esteratic site whilst the choline molecule is held in the anionic site. The anionic site in the *T. californica* AChE is hydrophobic in nature (Sussman *et al.*, 1991) and mutation studies on AChE have shown that a tryptophan residue at position 86 in the protein is vital for the binding of the quaternary ammonium of acetylcholine (Shaffman *et al.*, 1992; Ordentlich *et al.*, 1993).

1.4.3. LOCALISATION AND EXPRESSION

Vertebrate non-specific esterases are at their highest concentrations in liver, kidney and brain where almost all the esterolytic activity at neutral and alkaline pH is due to carboxylesterases (Kao *et al.*, 1985a). The carboxylesterases are mainly located in the microsomal and mitochondrial fractions showing that they are intracellular. Many of the rat liver carboxylesterases are microsomal (Heymann, 1980; Takagi *et al.*, 1988; Gaustad *et al.*, 1992; Robbi & Beaufay, 1988; Kluge *et al.*, 1990). Other rat carboxylesterases have been found to be located in serum (Alexson *et al.*, 1994), adipose tissue (Tsuujita & Okuda, 1992), pancreas (DiPersio & Hui, 1993) and lung (Gaustad *et al.*, 1991). In the mouse, carboxylesterases have been found that are specific to red blood cells, serum, and kidney and are not found in other tissues (Peters, 1982) suggesting that these have specific physiological roles.

The most studied of the insect esterases are those of *Drosophila* and the location of all soluble esterases of this insect have been determined (Healy *et al.*, 1991). Est6 from *D. melanogaster* and EstS from *D. virilis* are expressed most in the anterior ejaculatory duct (Scott *et al.*, 1984) and the ejaculatory bulbs of the adult male (Sergeev *et al.*, 1993), although Est6 is expressed in other tissues of both sexes. Deletion mutants of the 5' end of the Est6 gene have revealed which regions of the gene are necessary for the spatial, temporal and sex specific expression of the enzyme (Ludwig *et al.*, 1993). The carboxylesterase AChE is anchored to the plasma membrane and is almost entirely located in the brain of *D. melanogaster* (Mutero & Fournier, 1992). In *L. cuprina*, various esterase isozymes have been shown to be

expressed in different tissues and at different stages of the life cycle (Parker *et al.*, 1991). Isozymes E1 and E2 were found mainly in the insect's head, E1 being present from the larval stage onwards but E2 being present only in the pupa and adult. E3 was detected at highest levels in the Malpighian tubules and E4 in the haemolymph, both esterases being present at all life stages. Gut tissues of *C. tarsalis* have been found to contain the highest levels of carboxylesterase activity when compared to that detected in head thorax and the rest of the abdomen (Matsumura & Brown, 1963). An esterase, designated SG-EST, has been detected in the salivary gland of *Ae. aegypti* but no other tissues of the mosquito (Argentine & James, 1995). This esterase was secreted in saliva when both sugar-feeding and blood-feeding.

1.4.4. IMMUNOLOGICAL RELATIONSHIPS

A study of the immunochemical interrelationship between carboxylesterase isozymes in the mouse has found that the 14 isozymes isolated fall into two immunologically distinct groups (Ronai *et al.*, 1985). This separation on the grounds of immunological characteristics parallels the clustering of the genes coding for the esterases on chromosome 8 (Deimling *et al.*, 1983) with two separate clusters being detected. Esterases from within each cluster cross-reacted only with antisera raised against themselves or against other esterases within the cluster. The esterases within each cluster were thus apparently more closely related to each other phylogenetically than to isozymes from the other cluster. This property was not evident from the biochemical characteristics of these esterases. From these results

it was proposed that the two esterase clusters on chromosome 8 of the mouse each form a multigene family, the multiplicity of the esterase isozymes having arisen as a result of repeated duplication of an ancestral gene (Ronai *et al.*, 1985). An antiserum raised against rat microsomal acyl-CoA thioesterase was found to discriminate between this esterase and other esterases active on *o*-nitrophenyl acetate (Mentlein *et al.*, 1984). However, when an immunocolumn was prepared coupling the anti-(microsomal thioesterase) IgG to CNBr-activated Sepharose, the column was found to bind 80-90 % of total carboxylesterase activity (Alexson *et al.*, 1993). The additional bound carboxylesterases could only be eluted and separated from the thioesterase by a high salt wash. This indicated that, whilst the affinity of the immunocolumn was highest for the thioesterase, it also had a high affinity for other carboxylesterases. The antiserum also recognised carboxylesterases from porcine and rabbit liver.

An antiserum raised against the *Myzus persicae* E4 esterase has been used to show the presence of increased amounts of this esterase in insecticide resistant compared to susceptible aphids (Devonshire *et al.*, 1986a). This antiserum was used successfully to screen field populations for the elevated esterase E4 mechanism (French-Constant & Devonshire, 1988). The same antiserum was also observed to cross-react with a resistance-associated elevated esterase in the aphid *Phorodon humuli* (Devonshire *et al.*, 1986a).

An investigation of the cross-reactivity of antisera raised against *Culex* esterases revealed that, on western-blots of crude homogenates of *Culex* mosquitoes

containing various esterases α and β , an antiserum raised against denatured esterase Est β 1¹ purified from the Californian TEM-R strain of *C. quinquefasciatus* cross-reacted only with other esterases β (Est β 1¹ and Est β 2 in *C. quinquefasciatus*, and esterase B₃ in *C. tarsalis*), and an antiserum raised against denatured esterase Est α 1 purified from the French S54 strain of *C. pipiens* reacted only with other esterases α (Est α 1 in *C. pipiens*, Est α 2 in *C. quinquefasciatus*, and esterase A₃ in *C. tarsalis*) (Mouches *et al.*, 1987). This study used the 4-chloro-1-naphthol detection method. Poirie *et al.* (1992) examined the cross-reactivity of the TEM-R Est β 1¹ antiserum with immunoblots of crude homogenates of the VIM and CYPRUS strains, both containing esterases Est α 4 and Est β 3, and also found it to cross-react only with Est β 3. Proteins immunologically related to the Est β 1¹ were detected in *Ae. aegypti*, *Myzus persicae* and *M. domestica* (Mouches *et al.*, 1987). However, no positive reaction was observed between the Est β 1¹ antiserum and proteins in either *An. albimanus* or *An. stephensi* (Beyssat-Arnaouty *et al.*, 1989). A dot-blot assay was developed using the Est β 1¹ antiserum that enabled the presence of elevated esterases β associated with OP resistance to be diagnosed in field collections of *C. quinquefasciatus*, *C. pipiens* and *C. tarsalis* (Beyssat-Arnaouty *et al.*, 1989), and it was suggested that the same assay could be used with the antiserum raised against the S54 Est α 1 (Mouches *et al.*, 1987) to detect elevated esterases α .

Using the dye 3,3'-diaminobenzidine tetrahydrochloride for detection of cross-reactivity, a polyclonal antiserum raised against native esterase Est α 2¹ purified from the PelRR strain of *C. quinquefasciatus* was shown to cross-react with Est α 2¹ but not with Est β 2¹ from the same strain (Jayawardena, 1992). The antiserum was also

observed to cross-react strongly with the *Culex* AChE. More recently, the highly sensitive ECL (enhanced chemiluminescence) detection system was used to detect cross-reactivity of the PelRR Est α 2¹ antiserum with esterases α and β in strains of *C. quinquefasciatus* in western-blots and, with the purified esterases, in dot-blots (Karunaratne *et al.*, 1993; Karunaratne, 1994; Karunaratne *et al.*, 1995b). In contrast to previous studies, cross-reactivity was detected between this esterase α antiserum and esterases β , in both western blots and dot blots, although this cross-reactivity was lower than with the esterases α . A moderate cross-reactivity was detected between the antiserum and the malathion carboxylesterase of *An. stephensi*. This is in contrast to the findings of Beysatt-Arnaouty *et al.* (1989) who found no cross-reactivity of the TEM-R Est β 1¹ antiserum and proteins of this same species. A strong immunological cross-reactivity was seen between the Est α 2¹ antiserum and rabbit liver esterase (1-10 fold less reactive than with Est α 2¹), whilst cross-reactivities with electric eel AChE, bovine AChE, human AChE and horse butyrylcholinesterase were about 10⁴, 10², 10³ and 10⁴ fold respectively, less than that with Est α 2¹ (Karunaratne *et al.*, 1995b).

1.4.5. GENETICS

There have been many studies documenting the inheritance and linkage relationship of mammalian esterases. From these it is clear that mammalian esterases are highly polymorphic and map to many different loci. Most of the mouse carboxylesterase genes are located on chromosome 8 (Peters, 1982; Ronai *et al.*, 1985), whilst human carboxylesterase genes are mainly located on chromosome 16 (Kroetz *et al.*, 1993),

rabbit carboxylesterase genes on chromosome 6 (Zutphen *et al.*, 1987) and rat carboxylesterase genes on chromosome 5 (Alexson *et al.*, 1994). In the mouse, rabbit and rat, many of the loci for carboxylesterases have at least two alleles, with loci in all three mammals forming two distinct clusters (Peters, 1982; Ronai *et al.*, 1985; Hedrich & Deimling, 1987; Zutphen *et al.*, 1987; Deimling & Gaa, 1992). The two clusters of genes on chromosome 8 of the mouse code for proteins that are immunologically distinct suggesting that the two clusters arose from a duplication of an ancestral gene, the resultant genes then undergoing subsequent duplications to form the clusters (Ronai *et al.*, 1985). Using the sequence of six esterase genes from *D. melanogaster*, *Torpedo californica*, rat, rabbit and human and the COOH-terminal portions of two thyroglobulin genes from rabbit and cow, Takagi *et al.* (1991) has constructed a phylogenetic tree, the appearance of which suggests that these genes have arisen from a series of duplications starting with an ancestral gene that existed before the evolution of the vertebrates.

The *D. melanogaster* esterase gene *Est6* maps to chromosome 3 (Scott *et al.*, 1984). High resolution electrophoresis and comparison of deduced amino acid sequences has revealed the existence of 10 allozymes of this esterase (Cooke & Oakeshott, 1989). In addition, a second esterase gene, *EstP*, has been discovered which has a high degree of similarity to *Est6*, prompting the hypothesis that the two arose through a gene duplication (Collet *et al.*, 1990). Comparison of the nucleotide and deduced amino acid sequences of *Est5B* in *D. pseudoobscura* and *Est6* suggests that these too arose from duplication of an ancestral gene (Brady *et al.*, 1990). Two esterase genes, *Est4* and *Est5*, in *D. mojavensis* and *D. arizonensis* were mapped to

chromosome 2 in both species with the genes being at separate structural loci at a distance of less than 0.16 recombination units from each other (Zouros *et al.*, 1982). The two esterases are functional as dimers of differing electrophoretic mobility. Monomeric units from each of the genes have been observed to form a functional heterodimer of intermediate mobility. This, together with their close proximity on chromosome 2 prompted the hypothesis of gene duplication from an ancestral gene (Zouros *et al.*, 1982). A cluster of esterase genes has been mapped to chromosome 3R of *D. melanogaster* (Spackman *et al.*, 1994), these including homologs of esterase genes in the sheep blowfly *L. cuprina* which also form a cluster of a malathion carboxylesterase and two other esterases associated with resistance (Smyth *et al.*, 1994). In *Musca domestica*, an esterase gene associated with resistance to trichlorphon (an OP) and pyrethroids has been mapped to chromosome 2 (Sawicki *et al.*, 1984).

In the mosquito *An. stephensi*, genetic analyses have shown that the malathion carboxylesterase is on linkage group III, about 24 crossover units from a dieldrin resistance gene and 38 crossover units from the recessive mutant *diamond palpus* (Rowland, 1985). Three-point crosses, using the two insecticides in turn, indicated a gene sequence of *dieldrin resistance-malathion carboxylesterase-diamond palpus*. A locus for refractoriness in the malaria vector *An. gambiae*, affecting the ability to encapsulate the malaria parasite *Plasmodium cynomolgi* has been shown to be associated with two tightly linked esterase loci, *Est1*, a cholinesterase, and *Est2*, a carboxylesterase on chromosome 2 (Crews-Oyen *et al.*, 1993). In addition, chromosomal inversions on the left arm of chromosome 2 were inseparably

associated with different alleles at the two esterase loci. The resistance-associated esterase genes in *C. tritaeniorhynchus* are on chromosome II (Takahashi & Yasutomi, 1987), whilst in the *C. pipiens* complex most are on chromosome III (Pasteur *et al.*, 1981a). The possible exception is for the elevated *Est* β 1 gene which, from the results of *in situ* hybridization of a ³H-labelled cDNA probe of the TEM-R *est* β 1¹ to TEM-R chromosomes, was assigned to chromosome II (Nance *et al.*, 1990). However, in Wirth *et al.* (1990) the disjunction between *est* β 1¹, *est* α 2 and *est* β 2, places *est* β 1¹ on chromosome III. Linkages between the presence of esterases α and esterases β have been reported for elevated *Est* α 1 and a non-elevated *Est* β (*Est*-2⁰⁶⁴), for elevated esterases *Est* α 2 and *Est* β 2 (Wirth *et al.*, 1990), elevated *Est* α 4 and *Est* β 3 (Poirie *et al.*, 1992) in *Culex pipiens*, as well as for elevated esterases A3 and B3 in *Culex tarsalis* (Prabhaker *et al.*, 1987). The linkage map for *est* β 2, *est* α 2 and *est* β 1¹ suggests that they are located 2.9 and 3.9 centimorgans apart respectively (Wirth *et al.*, 1990), whilst *est* α 4 and *est* β 3 are 0.8 centimorgans apart (Poirie *et al.*, 1992). Despite the differences in the separation of the esterase α and β genes, and the placing of *est* β 2 and *est* β 1¹ loci at different positions on the chromosome (Wirth *et al.*, 1990), it has been proposed that all the loci coding for the esterases α are homologous and that all the loci coding for the esterases β are homologous (Poirie *et al.*, 1992).

1.4.6. MOLECULAR BIOLOGY

The gene and/or cDNA sequences of a large number of carboxylesterases have been obtained from a diverse range of organisms. These include sequences for

AChE from *D. melanogaster* (Hall & Spierer, 1986), *An. stephensi* (Hall & Malcolm, 1991), the electric fish *Torpedo californica* (Schumacher *et al.*, 1986), and man (Soreq *et al.*, 1990), a juvenile hormone esterase (Hanzlik *et al.*, 1989) and non-specific esterases from the rat (Takagi *et al.*, 1988; Takagi *et al.*, 1991; Robbi *et al.*, 1990), man (Munger *et al.*, 1991; Riddles *et al.*, 1991), the aphid *Myzus persicae* (Field *et al.*, 1993), *Drosophila* (Cooke & Oakeshott, 1989; Brady *et al.*, 1990; Collet *et al.*, 1990), and the mosquito *Culex quinquefasciatus* (Mouches *et al.*, 1990; Vaughan *et al.*, 1995; Vaughan & Hemingway, 1995).

Myers *et al.* (1988) proposed that, on the basis of their catalytic triad of serine, aspartic acid and histidine, the carboxylesterases could be placed into a larger group of enzymes all having this same triad. In *Torpedo californica* AChE, the nucleophilic serine²⁰⁰ lies within a narrow active site gorge and is with this residue that OPs bind covalently (Sussman *et al.*, 1991). Mutation of the active site serine to a cysteine residue impairs the function of the enzyme, whilst mutation to valine completely inactivates it (Gibney *et al.*, 1990). Replacement of the histidine residue at position 440 by glutamic acid eliminated activity, whereas the mutation of the His⁴²⁵ only slightly reduced activity (Gibney *et al.*, 1990), suggesting that it is the His⁴⁴⁰ that forms part of the catalytic triad. Studies of the catalytic triad of human AChE, including mutation studies, have shown that, contrary to the proposal of Myers *et al.* (1988), it is a glutamic acid residue, not an aspartic acid residue, that forms part of the triad (Shafferman *et al.*, 1992). In the large group of enzymes including carboxylesterases, lipases and related proteins, the acid residue in the catalytic triad is almost always glutamate (Cygler *et al.*, 1993). However, in *Heliothis* juvenile

hormone esterase the glutamate is replaced by a glutamine (Hanzlik *et al.*, 1989), and in *Drosophila* esterases 6 and P by a histidine (Oakeshott *et al.*, 1987; Collet *et al.*, 1990).

Carboxylesterases have, in common with carboxypeptidases, dieneacetone hydrolases, dehalogenases and lipases, a structure known as the α/β hydrolase fold (Ollis *et al.*, 1992). The core of each of these enzymes is similar, each having eight β -sheets connected by α -helices with the active site nucleophile located between β -strand 5 and α -helix C. The identity of the members of the catalytic triad of this group of enzymes varies, the only conserved residue being the histidine. It has been suggested that the α/β hydrolase fold enzymes have diverged from a common ancestor and that, whilst the members of the group show a lack of sequence similarity, they have evolved so as to preserve the positions of the key catalytic components (Ollis *et al.*, 1992). Based on the knowledge of the three-dimensional structure of *T. californica* AChE (Sussman *et al.*, 1991) and a lipase from the fungus *Geotrichum candidum* (Schrag & Cygler, 1993), and using the deduced amino acid sequences of some members of the α/β hydrolase fold enzymes and related proteins, Cygler *et al.* (1993) produced a multi-alignment which showed that 24 residues were invariant in the hydrolases, and an additional 49 well conserved. The conserved residues include the active site, disulphide bridges, and residues in the core of the proteins, with the most invariant residues being located at the edges of the secondary structural elements.

In both the aphid *Myzus persicae* and mosquitoes of the *C. pipiens* complex, OP

resistance has been shown to be associated with an elevation in non-specific esterases that sequester the insecticides (Needham & Sawicki, 1971; Curtis & Pasteur, 1981; Peiris & Hemingway, 1990b). Elevation of proteins may arise through several different mechanisms; by stabilisation of mRNA coding for the protein, increased transcription of the gene, increased translation of the mRNA or by gene amplification. Several of these mechanisms may act simultaneously. A cDNA clone of the *Myzus persicae* esterase E4 and a partial cDNA of the *Culex quinquefasciatus* esterase *estB1*¹ from the TEM-R strain were used in separate studies to show that, in both insects, elevation of esterases is due to gene amplification (Field *et al.*, 1988; Mouches *et al.*, 1986). Different degrees of amplification were shown to be correlated with the activity of the esterases in *Myzus persicae* and *C. quinquefasciatus* and, in the case of *Myzus persicae*, also with the level of resistance (Field *et al.*, 1988; Raymond *et al.*, 1989). In the *C. quinquefasciatus* laboratory strain TEM-R, which has been selected for many generations with temephos, the *estB1*¹ gene was estimated to be amplified about 250-fold, when compared to the OP susceptible S-Lab strain (Mouches *et al.*, 1986). This amplification is inherited through the germline and is thus stable (Nance *et al.*, 1990). In *Myzus persicae*, tandem duplication of the gene(s) has led to an exponential increase in the number of genes with a concomitant increase in OP resistance (Devonshire & Sawicki, 1979). Southern blots of *EcoRI* digested genomic DNA of the OP resistant PelRR strain possessing *estα2*¹ and *estβ2*¹, and of the susceptible PelSS strain possessing *estα3* and *estβ1*³ hybridised with PelRR *estα2*¹ and *estβ1*¹ cDNA probes have shown that both the esterases in PelRR are amplified (Vaughan *et al.*, 1995; Vaughan & Hemingway, 1995). The amplified esterase E4 related sequences were found to be

highly methylated (Field *et al.*, 1989). This methylation was lost when the aphid clones were not under insecticide selection pressure, the loss of methylation coinciding with a loss of elevated E4 mRNA whilst retaining the amplified sequences.

Gene amplification is found frequently in biology. Examples range from amplification of proto-oncogenes as a causative agent in cancer malignancy (The Lancet, 1987), to stage specific amplification of chorion genes in *D. melanogaster* (Kelley *et al.*, 1986; Komitopoulou *et al.*, 1986; Heck & Spradling, 1990), amplification of rDNA genes during oogenesis in *Xenopus laevis* (Stark & Wahl, 1984), and amplification of the dihydrofolate reductase gene in methotrexate resistance in cultured murine cells (Schimke, 1984). The cause of amplification events and the mechanism(s) by which it occurs are as yet poorly understood. In *Drosophila*, several cis-acting elements have been identified that are thought to be important for amplification. Short, apparently essential amplification-control elements (ACE)^{occur} within the chorion clusters of the X ($ACE-1$) and third ($ACE-3$) chromosome (Kalfayan *et al.*, 1985; Orrweaver *et al.*, 1989). However, with deletion of the $ACE-3$ region from the third chromosomal cluster, low-level amplification was still seen (Swimmer *et al.*, 1989) showing that, whilst this element is important in amplification, it is not essential. In *C. quinquefasciatus*, the amplification unit or 'amplicon' contains not only the *estB1*¹ gene, but also truncated copies of transposable Juan-like elements (Mouches *et al.*, 1990). There are full-length copies of Juan-C elements throughout the *C. pipiens* genome (Agarwal *et al.*, 1993) which, from sequence analysis, belong to the long interspersed repetitive element (LINE)

The MRES strain of *C. quinquefasciatus* is an OP and carbamate resistant strain from Havana, and was collected from the Quibu River district in 1986. Selection of fourth instar larvae at the 85%-95% mortality level resulted in resistance to malathion of more than 1000-fold. The strain is homozygous for both an altered AChE mechanism and an elevated Est β 1-based mechanism of insecticide resistance.

superfamily of retrotransposable ubiquitous DNA elements. LINEs have been found dispersed in mammals, *Drosophila*, *Aedes*, trypanosomes, *Neurospora* and *Zea* (Nocera & Sakaki, 1990; Bucheton *et al.*, 1986; Fawcett *et al.*, 1986; Mouches *et al.*, 1992). It has been suggested that the Juan element in the TEM-R *estB1*¹ amplicon maybe linked to the amplification process (Mouches *et al.*, 1991). However, this is unlikely given that the element is truncated. To date, no direct evidence has been reported of the involvement of LINE elements in the evolution of insecticide resistance mechanisms. It has yet to be shown whether the amplicon containing the *estα2* and *estβ2* genes contains any LINE elements.

The nucleotide sequence and predicted amino acid sequence of the *Culex* TEM-R *estB1*¹, MRES *estB1*², PelRR *estB2*¹, PelSS *estB1*³ (a partial-length cDNA) and PelRR *estα2*² have all been published (Mouches *et al.*, 1990; Vaughan *et al.*, 1995; Vaughan & Hemingway, 1995) together with the predicted amino acid sequence of a partial-length *estβ2* from the SeLax strain (Raymond *et al.*, 1991). All the sequences, except the Selax partial-length *estβ2*, for which the predicted amino acid sequence did not extend back to the active site serine residue, had a catalytic triad of Ser¹⁹¹, Glu³²⁴ and His⁴⁴² [based on the alignment of Cygler *et al.* (1993)]. All the nucleotide sequences and deduced amino acid sequences of the *estβ* genes show a high level of identity (>95% at both levels) (Vaughan *et al.*, 1995). The level of identity between the *estα2*¹ and the *estβ2*¹ genes was 58.3% at the nucleotide level and 49.2% at the amino acid level (Vaughan & Hemingway, 1995). They share common intron/exon boundaries, sit together on the same amplicon and are more closely related to each other than to any other sequences held in the database

(Vaughan, 1995). These data together suggest that *est α 2*¹ and *est β 2*¹ arose originally from a duplication of a common ancestral gene. The TEM-R *est β 1*¹ and MRES *est β 1*² had a percentage identity at the amino acid level of only 96.1%, and the two genes were also shown to differ in their genomic DNA *Eco*RI restriction fragment size (Vaughan *et al.*, 1995) suggesting that these two electrophoretically identical esterases have been amplified independently or, if amplification has occurred only once, this did not happen recently. However, restriction maps of the *est β 2* gene from populations of different geographic origin were shown to be identical suggesting that amplification occurred only once and that mosquitoes possessing the amplified gene then migrated worldwide (Raymond *et al.*, 1991).

cDNAs of esterases E4 and FE4 from *Myzus persicae* have been isolated and sequenced (Field *et al.*, 1993). The cDNAs coded for proteins of 529 and 541 amino acids respectively, the difference in size being due to a single nucleotide substitution which results in the FE4 mRNA having an extra 36 nucleotides at the 3' end. Over the region of the FE4 cDNA corresponding with that of the E4 cDNA, there was an identity of 99% at the nucleotide level and 98% at the amino acid level. There are only 20 nucleotide differences, nine of which change the corresponding amino acid of which all are conservative for charge and six also conservative for polarity. E4 shows conservation of residues Ser¹⁹¹, Glu³¹⁸ and His⁴⁴⁰ corresponding to those in the catalytic triad *Culex* esterases *Est α 2*, *Est β 1*, *Est β 2* and many other carboxylesterases, lipases and related proteins (Mouches *et al.*, 1990; Vaughan *et al.*, 1995; Vaughan & Hemingway, 1995; Cygler *et al.*, 1993). Only one esterase gene equivalent to E4 has been found in susceptible populations of *Myzus persicae* and

the sequence identity of this esterase gene with those of the E4 and FE4 genes suggests that E4 and FE4 arose by a recent gene duplication (A.L. Devonshire, personal communication). Comparison of the of *Culex estB2*¹ and the E4 genes revealed that they share an identity of 22.9% at the nucleotide level and 31.0% at the amino acid level over a 500-amino acid region (Vaughan *et al.*, 1995; Vaughan & Hemingway, 1995) whilst the *Culex estA2*¹ and the E4 gene share an identity at the nucleotide level of 21.8% (Vaughan & Hemingway, 1995).

1.5. BACKGROUND AND AIMS OF THE PRESENT STUDY

1.5.1. BACKGROUND OF THE STUDY

Exposure of *C. quinquefasciatus* to OPs, both as a target in mosquito control campaigns and as a non-target in attempts to control other insect pests, has resulted in the selection of esterase-based mechanisms in populations worldwide. The co-amplified esterase genes *estA2* and *estB2* have been found in organophosphate resistant *C. quinquefasciatus* populations on at least three continents (Raymond *et al.*, 1991). In California, co-elevated *EstA2* and *EstB2* were detected in 1984 (Raymond *et al.*, 1987), and in Cuba in 1991, only *EstB1* having been detected in populations from both geographic locations prior to these dates (Pasteur *et al.*, 1981a; Bisset *et al.*, 1990). Restriction fragment length polymorphism (RFLP) patterns of the amplified *estB2* gene and its flanking regions from African, Asian and North American populations were found to be identical whilst those of the non-amplified alleles showed a high level of variability, thus prompting the hypothesis

that the *estβ2* gene has been amplified only once and has subsequently spread worldwide by migration (Raymond *et al.*, 1991). The observation that *C. quinquefasciatus* strains from different geographical areas which have only the elevated esterase Estα2/Estβ2-based mechanism of OP resistance actually have different cross-resistance spectra (Georghiou & Pasteur, 1978; Amin & Peiris, 1990; Hemingway *et al.*, 1990; Peiris & Hemingway, 1990a; Peiris & Hemingway, 1990b) suggested this hypothesis might be incorrect. In addition, it has been found that elevated esterases Estα2 and Estβ2 purified from strains of different geographic origin, and from sub-colonies of a single strain (Sri Lankan PelRR) selected with different insecticides, showed variation in their bimolecular rate constants (k_a s) with various insecticides tested and it was suggested that these esterases may either be different allelic forms of a single Estα2 and Estβ2, or mixtures of different allelic forms of these esterases (Karunaratne *et al.*, 1993; Jayawardena *et al.*, 1994; Kettermann *et al.*, 1993; Karunaratne, 1994). To explain this result and also the variation in cross-resistance spectra, it was hypothesised that the *estα2* and *estβ2* genes might have been co-amplified several times, different alleles of these genes being amplified at each occurrence (Kettermann *et al.*, 1993).

More recently, data have been reported supporting the former (Raymond *et al.*, 1991) hypothesis. Southern blots of PelRR and PelSS *Eco*R1 genomic digests were probed with cDNA fragments of the PelRR *estα2*¹ and *estβ2*¹ esterase genes (Karunaratne *et al.*, 1995a). The restriction fragment of the amplified *estβ2*¹ gene in PelRR was the same size as that reported by Raymond *et al.* for *estβ2* in other strains (Raymond *et al.*, 1991). However, the PelRR strain was also found to contain

non-amplified *est* α and *est* β genes. The RFLP band of the non-amplified *est* β was the same size as the *est* β 1³ gene in PelSS, but the non-amplified *est* α was slightly different in size to the *est* α 3 gene in PelSS suggesting the presence of a different *est* α allele. It is unlikely that the purification methodologies employed would have separated the expressed esterases of these genes from the elevated esterases. Therefore, it was thought possible that the variation in the k_a s of the enzyme preparations from PelRR and other strains having elevated Est α 2 and Est β 2 might be due to minor contamination with different non-elevated esterases (Karunaratne *et al.*, 1995a), and/or to different levels of amplification of *est* α 2 and *est* β 2 with concomitant variation in the proportion of non-elevated esterases present. Indeed, whilst only a single non-amplified *est* α and *est* β allele was detected in the PelSS strain (Karunaratne *et al.*, 1995a), restriction fragment analysis of non-amplified *est* α and *est* β alleles in field populations have shown a high level of polymorphism (Raymond *et al.*, 1991). Since the PelRR strain still contains non-amplified *est* α and *est* β alleles, it is possible that other non-amplified alleles might exist in other populations of *C. quinquefasciatus* having co-amplified *est* α 2 and *est* β 2. This might in turn have led to the observed variations in insecticide cross-resistance spectra.

Elevated Est β 1 is more restricted in distribution, being found in populations from California, Cuba, Central America and Asia (Pasteur *et al.*, 1981a; Bisset *et al.*, 1990; Georghiou, 1992). In contrast to the restriction fragment patterns for the amplified *est* β 2 genes in *C. quinquefasciatus* strains of different geographic origin, which were found to be identical (Raymond *et al.*, 1991; Karunaratne *et al.*, 1995a), the restriction fragment patterns for two amplified *est* β 1 genes, *est* β 1¹ in the

Californian TEM-R strain (Mouches *et al.*, 1990) and *estβ1*² in the Cuban MRES strain, were found to differ (Vaughan *et al.*, 1995). Moreover, on sequencing these two *estβ1* cDNAs, they were found to differ at both the nucleotide and amino acid levels, homologies being 70.5% and 96.1% respectively (Vaughan *et al.*, 1995). On the basis of these data it was hypothesised that the genes coding for the two electrophoretically identical esterase Estβ1s from California and Cuba were allelic variants amplified independently (Vaughan *et al.*, 1995). This differs from the hypothesis for elevated Estβ2, namely that this arose from the a single amplification of an *estβ2* gene that has subsequently spread worldwide by migration (Raymond *et al.*, 1991).

To date, elevated Estβ1 has not been purified from any of the mosquito populations possessing it to determine unequivocally its role in insecticide resistance or to investigate possible variations in the biochemical characteristics of Estβ1 from populations of different geographic origin. In addition, Estα2 and Estβ2 have not been purified from any of the populations in the Americas now possessing them to investigate their biochemical characteristics and gain evidence as to whether they have arrived through migration of mosquitoes already possessing them or have occurred through novel amplification(s).

1.5.2. AIMS OF THE STUDY

The aims of this study are:

- 1) To purify and characterise, for the first time, an elevated Est β 1 from *C. quinquefasciatus* to reveal its properties and compare them with those of Est α 2 and Est β 2.
- 2) To establish the role of this esterase in insecticide resistance.
- 3) To investigate whether or not the elevated Est β 1 in strains of different geographic origin are functionally identical.
- 4) To purify co-elevated Est α 2 and Est β 2 from a population of *C. quinquefasciatus* originating from the Americas to investigate whether they have arrived by migration of mosquitoes possessing them, or have occurred through novel amplification(s).
- 5) To examine the immunological cross-reactivity of elevated esterases α and β with a polyclonal antiserum raised against elevated Est α 2¹ purified from the OP resistant PelRR strain (Karunaratne *et al.*, 1995a).

CHAPTER 2

Chapter 2

PURIFICATION AND CHARACTERIZATION OF THE CARBOXYLESTERASE Est β 1 FROM THE HABANA STRAIN.

2.1. INTRODUCTION

Use of organophosphorus insecticides (principally malathion, but also temephos and fenthion) for control of the mosquito *Aedes aegypti* in Cuba for 7 years up to 1986 selected for elevated carboxylesterase and altered acetylcholinesterase mechanisms of organophosphate resistance in the non-target mosquito *C. quinquefasciatus* which secondarily colonised the breeding sites typical of *Aedes* (Bisset *et al.*, 1990). These two factors together led to an increase in the biting nuisance of this mosquito which then itself became a focus for control of its number (Bisset *et al.*, 1990). Despite subsequent replacement of these insecticides by the pyrethroid cypermethrin in central Havana, these mechanisms remained at a high frequency, with less than 10% frequency of susceptible homozygotes, indicating that organophosphate resistance was still prevalent (Rodriguez *et al.*, 1993).

Prior to 1991, native PAGE undertaken to determine the identity of elevated esterases revealed two esterase bands (Bisset *et al.*, 1990). Using its substrate preference and relative mobility [according to the previous classification of (Raymond *et al.*, 1987)] compared with those found in other strains of *C. quinquefasciatus* and *C. pipiens* (see Villani *et al.*, 1983; Georghiou & Pasteur, 1978;

Callaghan, 1989) the major elevated esterase was tentatively named Est β 1. However, native PAGE undertaken subsequently revealed the presence in some populations from in and around Havana of other major esterase bands in addition to Est β 1. Of these, three of the esterases were tentatively named Est α 2, Est β 1 and Est β 2. The genes *est* α 2 and *est* β 2 are always found co-amplified and occur in mosquito populations on at least three continents (Raymond *et al.*, 1991). The occurrence of esterase Est β 1 is more restricted being found in populations from California, Cuba, Central America and Asia (Pasteur *et al.*, 1981a; Bisset *et al.*, 1990; Georghiou, 1992). As co-amplification of *est* α 2 and *est* β 2 is more common than amplification of *est* β 1, it was thought possible that the esterases coded for by these genes have a higher affinity for organophosphates than Est β 1 and hence confer a greater selective advantage in the presence of these insecticides. Indeed, the spread of *est* α 2 and *est* β 2 into populations in California (Raymond *et al.*, 1987) and into those in and around Havana seemed to strengthen this hypothesis. This study was therefore undertaken to determine the relative efficacy of the insecticide binding of esterase Est β 1 compared to other elevated and non-elevated *C. quinquefasciatus* esterases.

This chapter first shows the correlation between organophosphate and carbamate resistance, and results of biochemical assays for the detection of mechanisms of resistance indicating that elevated esterase and altered acetylcholinesterase mechanisms were operative in the Habana strain of *C. quinquefasciatus* from Cuba. It then details the further development of methodologies designed to purify esterases Est α 2¹ and Est β 2¹ from the Sri Lankan PelRR strain (Ketterman *et al.*,

1992; Jayawardena, 1992; Karunaratne *et al.*, 1993) to enable the first characterisation of an esterase EstB1.

2.2. MATERIALS AND METHODS

2.2.1. MOSQUITO STRAINS

The Habana strain was collected from Habana Vieja, a quarter of Havana City, Cuba, in 1991 and maintained without insecticide selection pressure. The PelSS strain used as a susceptible baseline in the bioassays and biochemical assays was derived from the 'Pel' strain by single family selection for low esterase activity (Peiris, 1989). The 'Pel' strain was collected in 1984 from Peliyagoda, Sri Lanka and was heterogeneous for OP resistance (Peiris & Hemingway, 1990b).

2.2.2. MOSQUITO COLONY MAINTENANCE

Adult mosquitoes were kept in cages of dimensions 45 x 45 x 45 cm and fed 20% (w/v) glucose solution *ad libitum*. Females were blood fed once a week on chicks confined in wire mesh cages and placed inside the mosquito colony cages overnight. Small white plastic tubs half filled with water were placed inside the cages four days later for egg laying. Adult cages were maintained under a 12 hour light/ 12 hour dark regime at 25°C and 80% relative humidity. Egg rafts were placed in large white plastic bowls filled with equilibrated tap water, 6 rafts per bowl. Bowls were

covered with elasticated netting to prevent any possible contamination from escapee female *Culex*. Larvae were fed *ad libitum* with a liver powder/yeast slurry. Upon pupation, the mosquitoes were transferred to white plastic emergence tubs which were placed into the adult cages. For mosquitoes to be used for ^{enzyme}purifications, larvae were mass-reared as previously described. On reaching the fourth instar larvae were strained, washed and blotted briefly on Whatman No. 1 filter paper (Whatman, U.K.) and layers of paper towelling. Larvae were weighed, snap frozen and then placed in a -70°C freezer until required for use.

2.2.3. CHEMICALS AND EQUIPMENT

Q-Sepharose Fast Flow, phenyl-Sepharose Fast Flow, PD-10 columns, Nap-5 columns, Superdex 200 (HR 16/60) FPLC column, PhastGel 8-25%, PhastGel IEF 4-6.5 and IEF 3-9, PhastSystem and Classic GP250 controlled FPLC system were purchased from Pharmacia, U.K. Hydroxylapatite, Prep-Cell 491, Mini-Protean II gel electrophoresis unit, pre-cast Mini-Protean II 4-20% gradient acrylamide gel. protein assay kit and molecular weight standards for SDS/PAGE were purchased from Bio-Rad, U.K. The *p*-chloromercuribenzoate-agarose was from Pierce (Chester, U.K.). Chemicals were purchased from Sigma, U.K., except where otherwise stated. Diethyl (dimethoxythiophosphorylthio) succinate (malathion, 97% pure) and its oxon analog (malaoxon, 87.5% pure), diethyl-4-nitrophenylphosphate (paraoxon, 97.4% pure), and 2-isopropoxyphenylmethylcarbamate (propoxur, 97% pure) were purchased from British Greyhound (Birkenhead, Merseyside, U.K.). The oxon analogues of *O,O*-diethyl *O*-3,5,6-trichloro-2-pyridyl phosphorothioate

(chlorpyrifos-oxon, analytical grade) and *O,O*-dimethyl *O'*-4-nitro-*m*-tolyl phosphorothioate (fenitrooxon, 98.3% pure) were gifts from Dow Elanco (Midland, MI, U.S.A.) and Sumitomo Chemical Co. (Osaka, Japan), respectively. α -Cyano-3-phenoxybenzyl (3-2-chloro-3,3,3,-trifluoroprop-1-enyl)-2,2-dimethylcyclopropanecarboxylate (lambda cyhalothrin, 81.2% pure), (*RS*)- α -cyano-3-phenoxybenzyl(1*RS*,3*RS*; 1*RS*,3*SR*)-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropanecarboxylate (cypermethrin) and 5-[2-(2-butoxyethoxy)ethoxymethyl]-4,5-methylenedioxy-2-propyltoluene (piperonyl butoxide, 87% pure) were gifts from Zeneca Public Health (Haslemere, Surrey, U.K.). SSS-Tributyl phosphorothioate (DEF) was a gift from Mobay, U.S.A.

2.2.4. LARVAL BIOASSAYS

Levels of insecticide resistance in the Habana strain were compared to the susceptible PelSS strain after larval bioassays. Bioassays were performed by exposing batches of 25 fourth instar larvae to known insecticide concentrations in 200 ml of distilled water (WHO, 1981). Malathion, propoxur and cypermethrin solutions were made up in acetone and 1 ml of the solution added to the water. A control was included with each assay by adding 1 ml of acetone alone. The action of two synergists, DEF and piperonyl butoxide (PB) were investigated by exposing the fourth instar larvae to 0.008 mg/l DEF or 5 mg/l PB for 4 hr prior to the addition of insecticide solution (Ranasinghe & Georgiou, 1979). At these concentrations there was no mortality with the synergist alone. For each bioassay at least five concentrations giving mortalities between 0 and 100% were tested, with

All biochemical assays were carried out in microtitre plates

two replicates for each concentration. After 24 hr insecticide exposure at $25 \pm 2^\circ\text{C}$ the larval mortalities were counted. Mortality data were subjected to probit regression with an unpublished programme written by C.J. Schofield (WHO, Geneva) based on the method of (Finney, 1971) and the lethal concentrations which gave 50% (LC_{50}) and 90% (LC_{90}) mortalities calculated.

2.2.5. BIOCHEMICAL ASSAYS FOR THE DETECTION OF THE ELEVATED ESTERASE, ALTERED ACETYLCHOLINESTERASE AND GLUTATHIONE S-TRANSFERASE INSECTICIDE RESISTANCE MECHANISMS

Biochemical assay techniques used for detection of the elevated esterase, altered acetylcholinesterase (AChE) and glutathione S-transferase (GST) insecticide resistance mechanisms were those developed by ffrench-Constant & Bonning (1989), Hemingway (1989), Hemingway *et al.* (1987) and Prapanthadara *et al.* (1993). The F_1 progeny of the field-collected material were subjected to these assays in order to determine the possible mechanisms involved in insecticide resistance in the Habana strain. Individual fourth instar larvae which had not been exposed to insecticide were homogenised in 200 μl of 0.02 M phosphate buffer, pH 7.2. For the esterase assay, the substrates α - and β -naphthyl acetate were used. Two 20 μl aliquots of homogenate were taken. To one replicate of homogenates, 200 μl of 0.3 mM α -naphthyl acetate working solution (1 ml of 30 mM α -naphthyl acetate acetone stock solution in 99 ml 0.02 M phosphate buffer [pH 7.2]) was added. To the other replicate, 200 μl of 0.3 mM β -naphthyl acetate working solution (prepared as for α -naphthyl acetate) was added. After incubation for 10 min at 37°C , the

reaction was stopped by the addition of 50 μ l of stain (150 mg Fast Blue B salt dissolved in 15 ml distilled water plus 35 ml 5% sodium lauryl sulphate in distilled water). Enzyme activity was measured at 570 nm as an endpoint in a UVmax plate reader (Molecular Devices, U.S.A.). Absorbance in each sample was measured against standard curves of serial dilutions of α - and β -naphthol, the products of the esterase hydrolysis, stained as described above. To assay for altered AChE, two aliquots of 40 μ l of homogenate were transferred to microtitre plate wells containing 135 μ l 1% (v/v) Triton X-100 in 0.1 M phosphate buffer, pH 7.8, and 10 μ l 0.01 M 5-5' dithiobis(2-nitrobenzoic acid) in 0.1 M phosphate buffer, pH 7.0. To one (control) aliquot, 25 μ l 0.01 M acetylthiocholine iodide in distilled water was added, and to the other (inhibited) fraction, 25 μ l of 0.01 M AChI with 0.01 M propoxur. Activity was measured at 405 nm for 5 min. For the assay of GST activity two replicates of 10 μ l of homogenate were added to microtitre plate wells containing 200 μ l 10 mM reduced glutathione, 3 mM 2,4-dinitrochlorobenzene, 4.8% (v/v) methanol in 0.1 M phosphate buffer, pH 6.5. GST activity was measured at 340 nm for 5 min. An extinction co-efficient of 5.76 mM⁻¹ (corrected for the path length) was used to convert the absorbances into moles. Protein concentration was assayed by incubating 10 μ l of homogenate, against various concentrations of bovine serum albumin as the standard protein, with 300 μ l of Bio-Rad protein assay solution [following the method of Bradford (Bradford, 1976)], for 10 min and absorption measured at 570 nm. All assays except for the esterase assay were performed at 22 °C.

2.2.6. ENZYME ASSAYS DURING PURIFICATION AND CHARACTERIZATION OF CARBOXYLESTERASE *EstB1*

Esterase activity was monitored during the purification with *p*-nitrophenyl acetate (*p*-NPA). A stock solution of 100 mM *p*-NPA was prepared in acetonitrile and stored at 4°C. This was diluted in 50 mM phosphate buffer, pH 7.4, to give a 1 mM working solution. 200 µl of working solution was added to 10 µl of sample and activity measured at 405 nm for 2 min in the UVmax plate reader. An extinction coefficient of 6.53 mM⁻¹ (corrected for a path length of 0.6 cm) was used to convert the absorbance into millimoles. The exceptions were for the elution of activity from the *p*-chloromercuribenzoate-agarose column and the Prep cell which was monitored by incubation with 1 mM *p*-nitrophenyl caproate under the same conditions, as this gave greater sensitivity with low concentrations of enzyme. Unless otherwise stated, all enzyme assays were performed at 22 °C. Protein concentration was estimated by the method of Bradford (1976) as previously described (see section 2.2.5.).

2.2.8. PURIFICATION OF CARBOXYLESTERASE *EstB1*

Carboxylesterase *EstB1* was purified by further development of the methods for purification of esterases *Estα2*¹ and *Estβ2*¹ (Ketterman *et al.*, 1992; Jayawardena, 1992; Karunaratne *et al.*, 1993). Unless otherwise stated, purification steps were performed at 4°C. Fourth instar larvae stored at -70°C were homogenised in 100 ml

of Bistris propane buffer (pH 7.0 with conductivity adjusted to 2.0 mS/cm) containing 25 mM DL-dithiothreitol (DTT). The homogenate was centrifuged at 10,000g for ten minutes and the supernatant filtered through Whatman No.1 filter paper. After adjusting pH and conductivity to their original values this was then applied to a Q-Sepharose Fast Flow column (4.4 x 4 cm) equilibrated with the same Bistris propane buffer but containing 15 mM DTT. The adjustment of pH and conductivity of the buffer facilitated the binding of esterase Est β 1 which, under the conditions of the original procedure, slowly eluted in the column wash following sample application. Esterases were eluted with a 10 bed volume salt gradient (0-0.5 M NaCl in Bistris propane buffer) and elution profiles determined for esterase activity, protein concentration and conductivity. The esterase activity eluted in one peak, salt concentration of the pooled fractions was adjusted to 3 M NaCl and the pH adjusted to pH 6.5 using dilute HCl. This sample was then applied to a phenyl-Sepharose Fast Flow column (2.2 x 8 cm) equilibrated with 25 mM Bistris propane buffer (pH 6.5) containing 15 mM DTT and 3 M NaCl. The absorbed esterase activity was eluted with 5 bed volumes of a decreasing salt gradient (3-0 M NaCl in the equilibration buffer) followed by isocratic elution once the gradient had ended. Esterase Est α 2 eluted from the phenyl-Sepharose column first, whilst esterases Est β 1 and Est β 2 co-eluted as a second activity peak. Fractions falling within this second peak were pooled, the concentration of DTT increased to 25 mM to protect the enzyme against oxidation, and dialysed against dry sucrose. Further de-salting and buffer exchange into the hydroxylapatite buffer was performed on PD-10 columns. The sample was then applied to a hydroxy apatite column (2.2 x 5.4 cm) equilibrated with 10 mM sodium phosphate buffer, pH 6.8, containing 15 mM

DTT and 50 mM NaCl. The esterase activity was eluted with a 5 bed volume gradient of the sodium phosphate buffer (10-200 mM, pH 6.8) containing no NaCl. The hydroxylapatite chromatography was performed at 22°C to avoid crystallization of sodium phosphate from the buffer. Esterase Est β 1 co-eluted with esterase Est β 2 from this column. The appropriate fractions were pooled, concentrated in Amicon centriprep 10 units, and applied to Nap-5 columns to exchange buffer into 0.1 M sodium phosphate buffer (pH 7.8) containing 10 mM EDTA. This sample was applied to a *p*-chloromercuribenzoate-agarose column (1.5 x 5 cm) equilibrated with the same buffer. The esterase activity was eluted with a 5 bed volume gradient of the equilibrating buffer and 20 mM sodium phosphate buffer, pH 6.8, containing 30 mM β -mercaptoethanol. Fractions with esterase activity were pooled and concentrated in Amicon centriprep 10 and centricon 10 concentrator units down to a volume of approximately 400 μ l. Blue marker [100 μ l 1% (w/v) solution of xylene cyanol FF marker dye in 5 ml 10% (v/v) glycerol in electrode buffer] was mixed with the concentrated enzymes in a ratio of 1:5 and the whole applied to a 9% acrylamide gel (12 cm gel height, 28 mm ID cell) in a Bio-Rad Model 491 Prep-Cell. The gel was run at 10 W constant power and the pure esterases eluted with the electrode buffer, 0.1 M Tris/borate, pH 8.0, with 2 mM EDTA, 10 mM DTT. Esterase Est β 1 was completely separated from esterase Est β 2 and eluted as a second activity peak from the Prep-Cell. The enzyme was concentrated, the buffer exchanged for 50 mM sodium phosphate buffer (pH 7.4) on Nap-5 columns and then stored in 50% (v/v) glycerol containing 25 mM DTT at -20 °C. Under these conditions the enzyme was stable over several months with no detectable decrease in specific activity. Esterase Est β 1 from several purifications, and from several

generations of mosquitoes, was obtained for use in the subsequent characterization studies to ensure that all results were reproducible.

2.2.9. CHARACTERIZATION OF CARBOXYLESTERASE *EstB1*

2.2.9.1. Polyacrylamide Gel Electrophoresis, Molecular Weight Determination and Determination of pI

Electrophoresis of native protein samples was performed in 7.5% acrylamide gels in 0.1 M Tris/borate buffer, pH 8.0, with 2 mM EDTA, by the method of Davis (1964) using a Bio-Rad Mini-Protean II electrophoresis unit. Individuals of the F_1 progeny of field-collected material were homogenised in 200 μ l of 0.02 M phosphate buffer (pH 7.2), 20 μ l aliquots of these homogenates mixed with blue marker (see section 2.2.8.) and then electrophoresed on 7.5% acrylamide gels. Esterases were visualised by incubation of gels with 0.04% (w/v) α - and β -naphthyl acetate, 0.1% (w/v) Fast Blue B in 100 mM sodium phosphate buffer, pH 7.4. Estimates of native M_r for the esterase *EstB1* were obtained by running samples of the purified esterase and Sigma standard proteins (M_r 14200-545000) on Phastgels (gradient 8-25%) on a Pharmacia PhastSystem for 1600 Vh at 4 °C. Gels were cut so that esterases could be visualised as previously described and standard proteins visualised by staining with Coomassie Blue R250. SDS/PAGE was performed with pre-stained Bio-Rad standard proteins (M_r 18500-106000) on a pre-cast Bio-Rad Mini-Protean II 4-20% gradient acrylamide gel (Laemmli, 1970). Coomassie Blue R250 stain was used to visualise proteins. For each SDS/PAGE gel, log molecular weight versus the

relative mobility of each standard was plotted to obtain a regression line. The molecular weight of esterase Est β 1 was determined using its relative mobility compared to the standards. For each gradient native PAGE gel log molecular weight was plotted against log relative mobility and molecular weight of the esterases Est β 1 determined as above. Isoelectric focusing was performed with Bio-Rad standard proteins for IEF (pI 4.65-7.8) on PhastGel IEF 3-9 and IEF 4-6.5 using the manufacturers optimised conditions. The pI of each standard protein was plotted versus its relative mobility to obtain a regression line. The pI of esterase Est β 1 was determined using its relative mobility compared to the standards.

2.2.9.2. Molecular Weight Determination by Gel Filtration Chromatography

A second estimate of native M_r of the esterase Est β 1 was obtained using gel filtration chromatography. Samples of the purified enzyme were applied to a pre-packed Pharmacia Superdex 200 (HR 16/60) column attached to a Pharmacia Classic GP250 controlled FPLC system. The column was equilibrated with 25 mM Bistris propane, 0.1 M NaCl, 10 mM DTT, pH 7.4, and samples run at 1 ml/min. The Superdex 200 column was calibrated using Sigma standard proteins for gel filtration chromatography. Log molecular weight versus $V_{\text{elution}}/V_{\text{void}}$ (where V = volume in ml) for each standard was plotted to obtain a regression line. The molecular weight of esterase Est β 1 was determined using its relative mobility compared to the standards. This was repeated for several runs.

2.2.9.3. Influence of effectors

Several compounds which may effect the enzyme activity and are commonly used to characterize different esterase types were used. Solutions of paraoxon (0.1 μ M and 0.1 mM), EDTA (1 mM), bis (*p*-nitrophenyl) phosphate (1 mM), and several metal ions (1 mM), including a series of transition metal ions, were prepared in 25 mM Bistris propane buffer, pH 7.4. Each effector was pre-incubated with the purified enzyme for 30 min at 22 °C. Esterase activity was measured with *p*-nitrophenyl acetate in the presence of each effector against activity of a control.

2.2.9.4. Kinetic constants

All specific activities are given in units/mg of protein, where one unit corresponds to the hydrolysis of 1 μ mol of substrate in 1 min under the assay conditions used. Kinetic constants were determined from at least three experiments for each substrate or insecticide using enzymes from several different purifications.

2.2.9.4.1 *Determination of the Michaelis Constant (K_m) and the Maximum Velocity (V_{max}) for the substrates*

The hydrolytic production of *p*-nitrophenol at different substrate concentrations was measured to determine the constants for *p*-NPA and *p*-nitrophenyl caproate (*p*-nitrophenyl hexanoate) using the assay as described previously. (see section 2.2.6).

Michaelis constant (K_m) and maximum specific activity (V_{max}) were calculated by non-linear regression using the ENZFITTER programme (by R.J. Leatherbarrow; Biosoft).

2.2.9.4.2. *Determination of the Kinetic Constant k_a for several insecticides*

For the determination of bimolecular rate constants (k_a), stopped time inhibition assays were performed using *p*-nitrophenyl acetate or *p*-nitrophenyl caproate as the substrate. An enzyme concentration which gave a reading of about 300 mODmin⁻¹ was used. Insecticide stock solutions were prepared in acetonitrile and diluted in 50 mM sodium phosphate buffer (pH 7.4) prior to the experiment. The purified EstB1 was incubated with a series of concentrations of the respective insecticide (acetonitrile concentration never exceeding 1% [v/v] in the incubation medium), and at various times aliquots taken and residual activity determined by measuring the rate of substrate hydrolysis. The activities were divided by control activities measured in the absence of insecticide. These data were then used to calculate the bimolecular rate constants for the formation of acylated enzyme (k_a) using the following equation (Aldridge & Reiner, 1972).

$$k_a = 1/[I]t \ln 100/X$$

where; [I] = insecticide concentration, t = time of pre-incubation of the enzyme and X = percentage remaining activity.

Where [I] could not be maintained in large excess due to the high affinity of the esterase for the insecticide, as was the case with chlorpyrifos-oxon and paraoxon, pseudo-first order kinetics could not be obtained. In these cases k_a values were determined in the presence of substrate by the method of Main & Dauterman (1963). This employs the equation

$$k_a = 2.303 / (t_2 - t_1)(1 - \alpha)[I] \log V_1 / V_2$$

$$\text{and, } (1 - \alpha) = K_m / (K_m + [S])$$

where; K_m = the Michaelis constant for the substrate, [S] = substrate concentration, V_1 = velocity after inhibition for time t_1 and V_2 is the velocity after inhibition for time t_2 . To minimise the effect of the reversible enzyme-substrate complex on the rate of acylation, the substrate concentration was adjusted so that the [substrate]/ K_m ratio was always less than 0.5 (Aldridge & Reiner, 1972).

Oxon analogues of the phosphorothionates were used rather than the phosphorothionates themselves as the oxons are much more potent inhibitors of *C. quinquefasciatus* esterases (Karunaratne, 1994), and because it is thought that toxicity *in vivo* is due to the oxons formed via oxidase metabolism of the thionates.

Other constants (k_2 , k_3 , K_a) were not determined, as previously it has been shown for esterases Est α 2 and Est β 2 that these constants did not correlate well with the observed organophosphate cross-resistance spectra (Karunaratne *et al.*, 1993).

2.3. RESULTS AND DISCUSSION

2.3.1. LARVAL BIOASSAYS

The bioassay of fourth instar Habana larvae indicates that there is a high level of malathion and propoxur resistance, with a low level of resistance to cypermethrin (TABLE 2.1 and FIGURES 2.1-2.3). The lower values for the slopes of the regression lines for the Habana strain, compared with the susceptible PelSS strain, suggests that it is heterogeneous with respect to resistance to all three insecticides. The Chi-square values showed that none of the data deviate significantly from a straight line response to any of the treatments.

Sampling in 1986 revealed organophosphate and carbamate resistance in several localities in and around Havana (Bisset *et al.*, 1990). At this time there was no evidence of pyrethroid resistance in any of the populations sampled. However, in common with samples from populations taken from 1990 onwards (Bisset *et al.*, 1991; Rodriguez *et al.*, 1993), the Habana strain shows low levels of resistance to pyrethroids.

The effects of pre-exposure of the Habana strain to the synergists DEF and PB are seen in FIGURE 2.1. DEF reduced the level of resistance to treatment with malathion, but the remaining level of resistance at both the LC_{50} and LC_{90} levels was still significantly higher than the susceptible PelSS strain. Pre-exposure with PB slightly increased the level of resistance compared to treatment with malathion

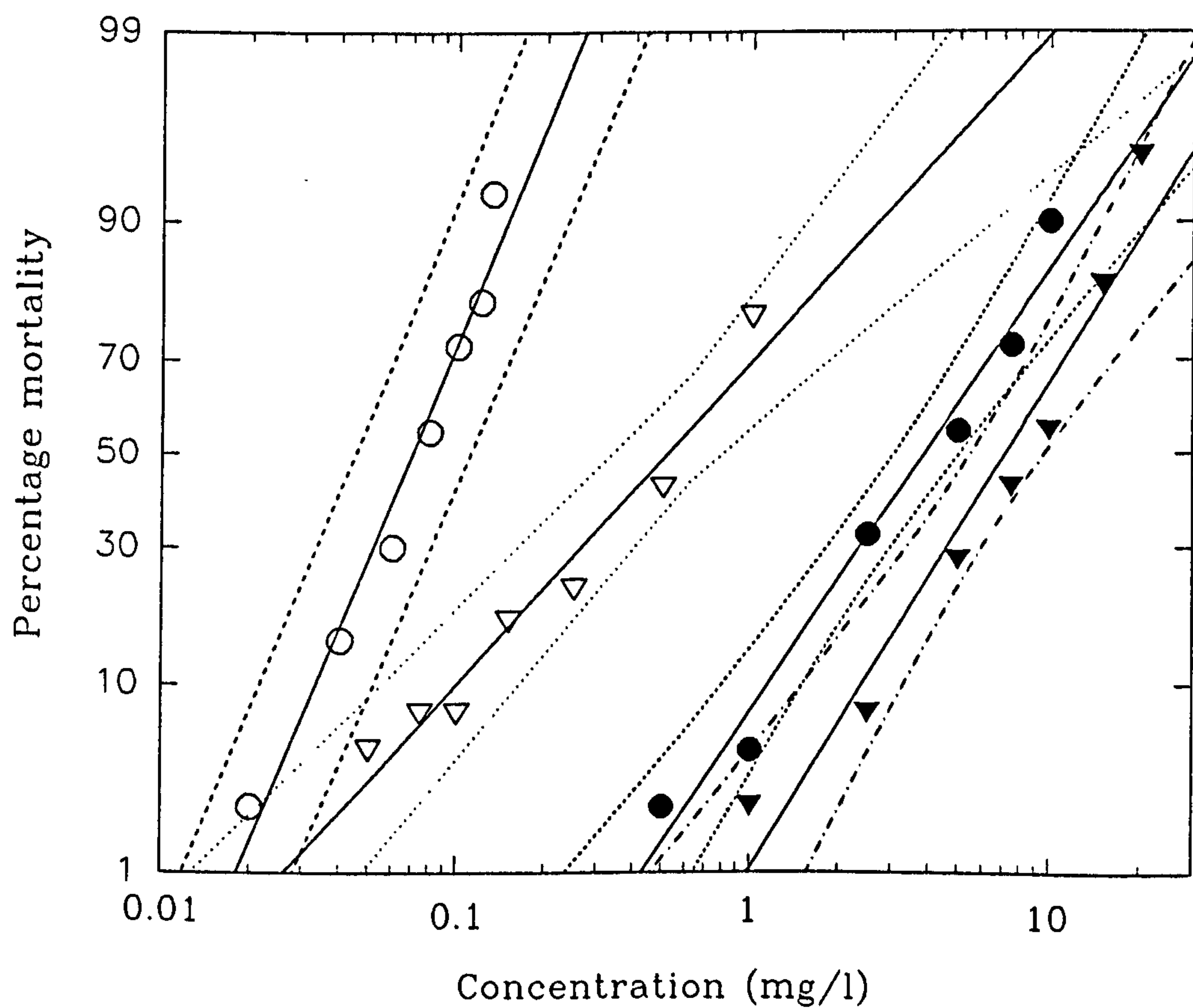
TABLE 2.1. Log-dosage probit mortality regression analysis for malathion (organophosphorus), propoxur (carbamate) and cypermethrin (pyrethroid) insecticides applied to fourth instar larvae of the susceptible PelSS and field-collected Habana strains of *C. quinquefasciatus*.

The calculated resistance ratios (RR) for Habana are also presented. Upper (U.L.) and lower (L.L.) 95% confidence limits are attached to each LC₅₀. Slopes of the probit lines are given as an indication of the relative homogeneity of data. No regression data had Chi-square values which deviated significantly from a straight line (P = <0.05).

Insecticide	Mosquito strain				
	PelSS		Habana		
	LC ₅₀ mg/l	LC ₉₀ mg/l	Slope	LC ₅₀ mg/l	Slope
Malathion	0.07	0.15	1.83	3.73	0.95
	U.L. 0.08 L.L. 0.06			U.L. 4.57 L.L. 3.05	
Propoxur	0.19	0.40	1.76	1.83	1.11
	U.L. 0.22 L.L. 0.17			U.L. 2.19 L.L. 1.54	
Cypermethrin	0.0024	0.006	1.56	0.009	1.13
	U.L. 0.028 L.L. 0.022			U.L. 0.011 L.L. 0.008	

FIGURE 2.1

Log-dosage mortality line for malathion in the field-collected Habana strain. The effect of the synergists DEF and PB on the response of the Habana strain are compared to the standard susceptible PelSS strain.



- PelSS malathion, 95% confidence limits
- Habana malathion, 95% confidence limits
- ▽ Habana DEF + malathion, 95% confidence limits
- ▼ Habana PB + malathion, 95% confidence limits

FIGURE 2.2

Log-dosage mortality line for propoxur in the field-collected Habana strain compared to the standard susceptible PelSS strain.

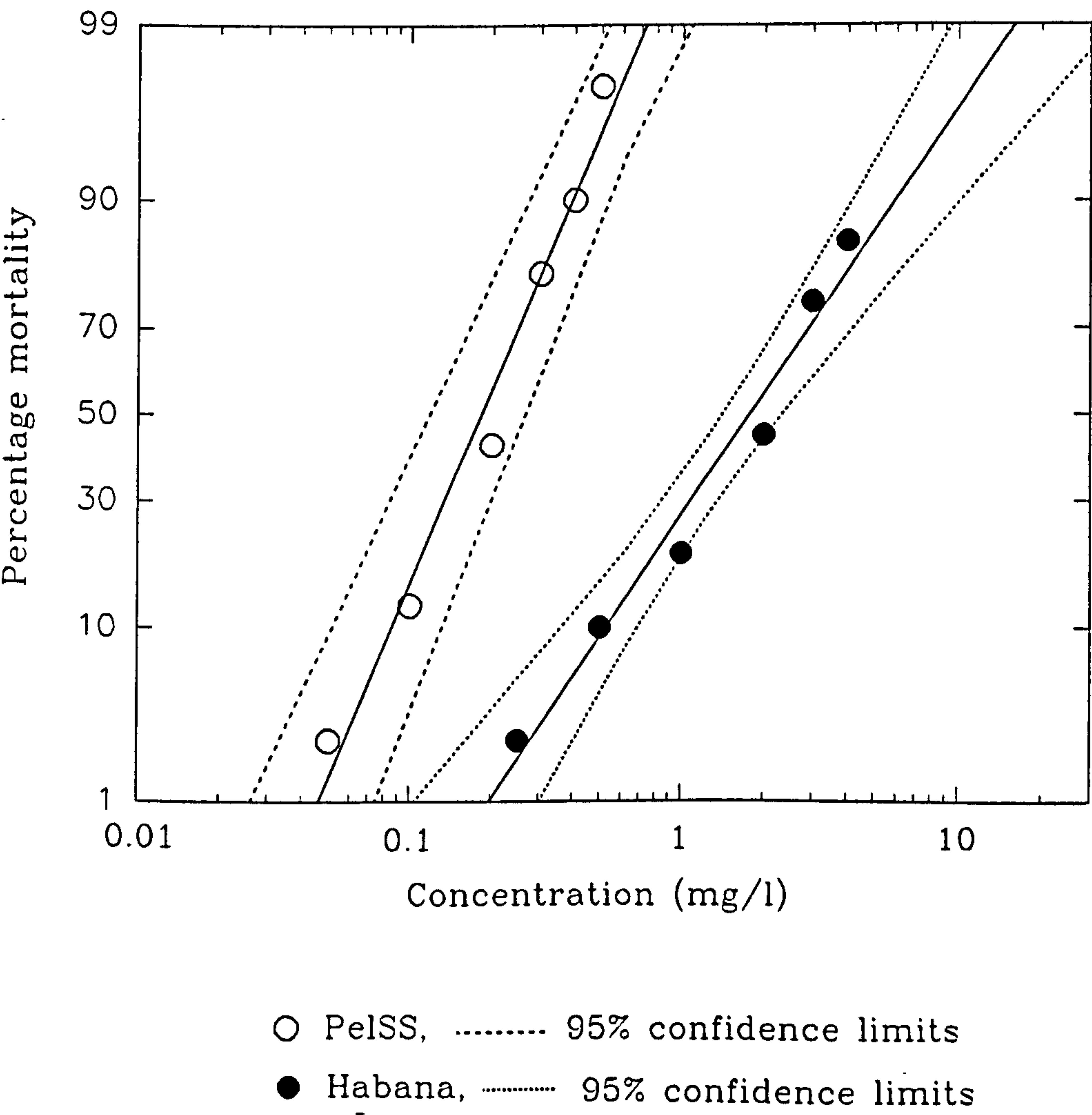
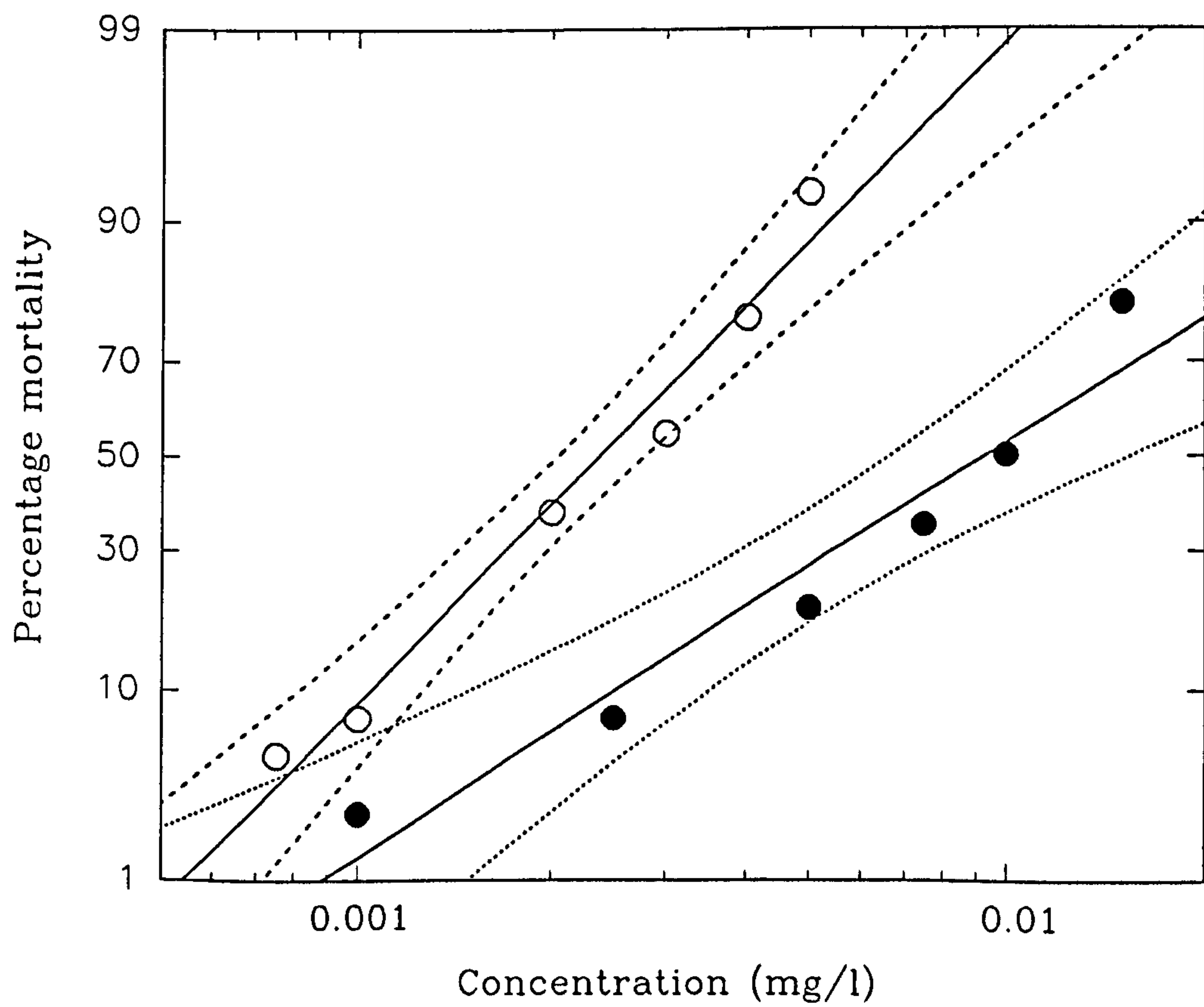


FIGURE 2.3

Log-dosage mortality line for cypermethrin in the field-collected Habana strain compared to the standard susceptible PelSS strain.



○ PelSS, 95% confidence limits
● Habana, 95% confidence limits

When the Hardy-Weinberg Law is applied to a locus carrying two alleles, p and q , the frequency of these alleles in a randomly mating population in Hardy-Weinberg equilibrium is:

$$(1) \quad p^2 + 2pq + q^2 = 1$$

For analysis of the frequency of the elevated esterase-based mechanism in the Habana population this can be re-written:

$$(2) \quad e^2 + 2eE + E^2 = 1$$

where e is the non-amplified esterase gene and E the amplified esterase gene. From FIGURE 2.4 it will be seen that the frequencies of the three phenotypes, low esterase activity (ee), high esterase activity (EE) and intermediate esterase activity (eE) can be distinguished. Using this data and formula (2) above, an approximate calculation was made of the elevated esterase-based resistance mechanism in the Habana population.

alone. This is consistent with a reduced level of conversion of the malathion, through the action of monooxygenases, to its more potent oxon analog. The synergist results^{are} consistent with esterase involvement in malathion resistance in combination with one or more further non-oxidase resistance mechanisms in the Habana strain.

2.3.2. BIOCHEMICAL ASSAYS FOR THE DETECTION OF THE ELEVATED ESTERASE, ALTERED ACETYLCHOLINESTERASE AND GLUTATHIONE S-TRANSFERASE INSECTICIDE RESISTANCE MECHANISMS

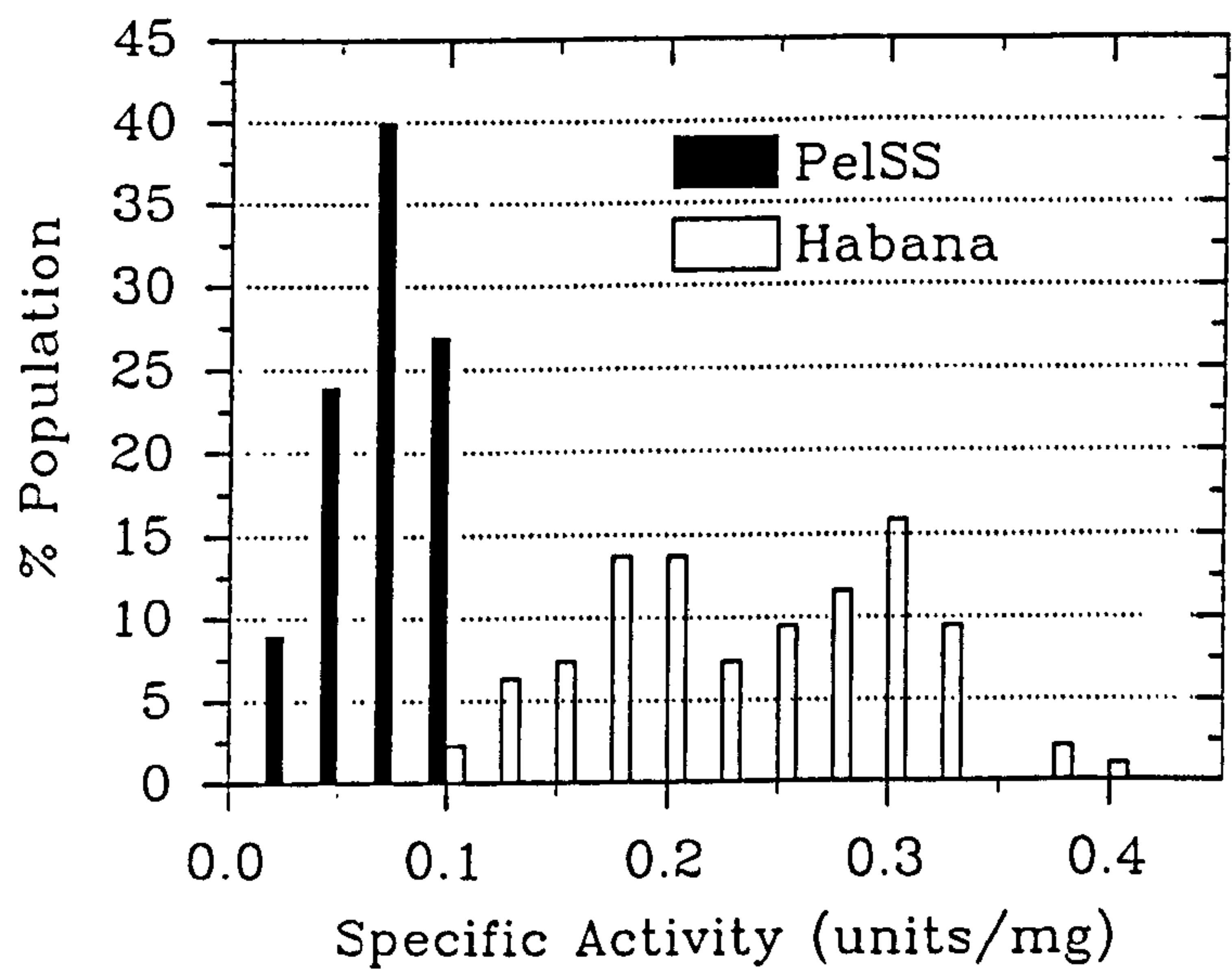
The results of the biochemical assay for esterase activity using the substrates α - and β -naphthyl acetate (FIGURE 2.4) confirm that there is a high level of general esterase activity in individuals of the Habana strain. Assuming that the Habana population was in Hardy-Weinberg equilibrium, and using the number of homozygous susceptibles, the frequency of the elevated esterase-based mechanism was calculated to be 0.85. This frequency is high compared with other Cuban field populations of *C. quinquefasciatus* where the elevated esterases frequencies generally have declined since the cessation of organophosphorus insecticide treatment for mosquito control in 1986. Two strains, QUIBU and SAN JOSE, obtained from different areas of Havana in 1986 and 1987, had frequencies for the elevated esterase mechanism of 0.85 and 1.0 respectively (Bisset *et al.*, 1990), whilst surveys of populations around Havana in 1990 revealed a decline in the frequency of this mechanism in some populations to 0.43 or below (Rodriguez *et al.*, 1993).

The esterase activity in 95 individual fourth instar larvae of the PelSS and Habana strains was determined with both substrates

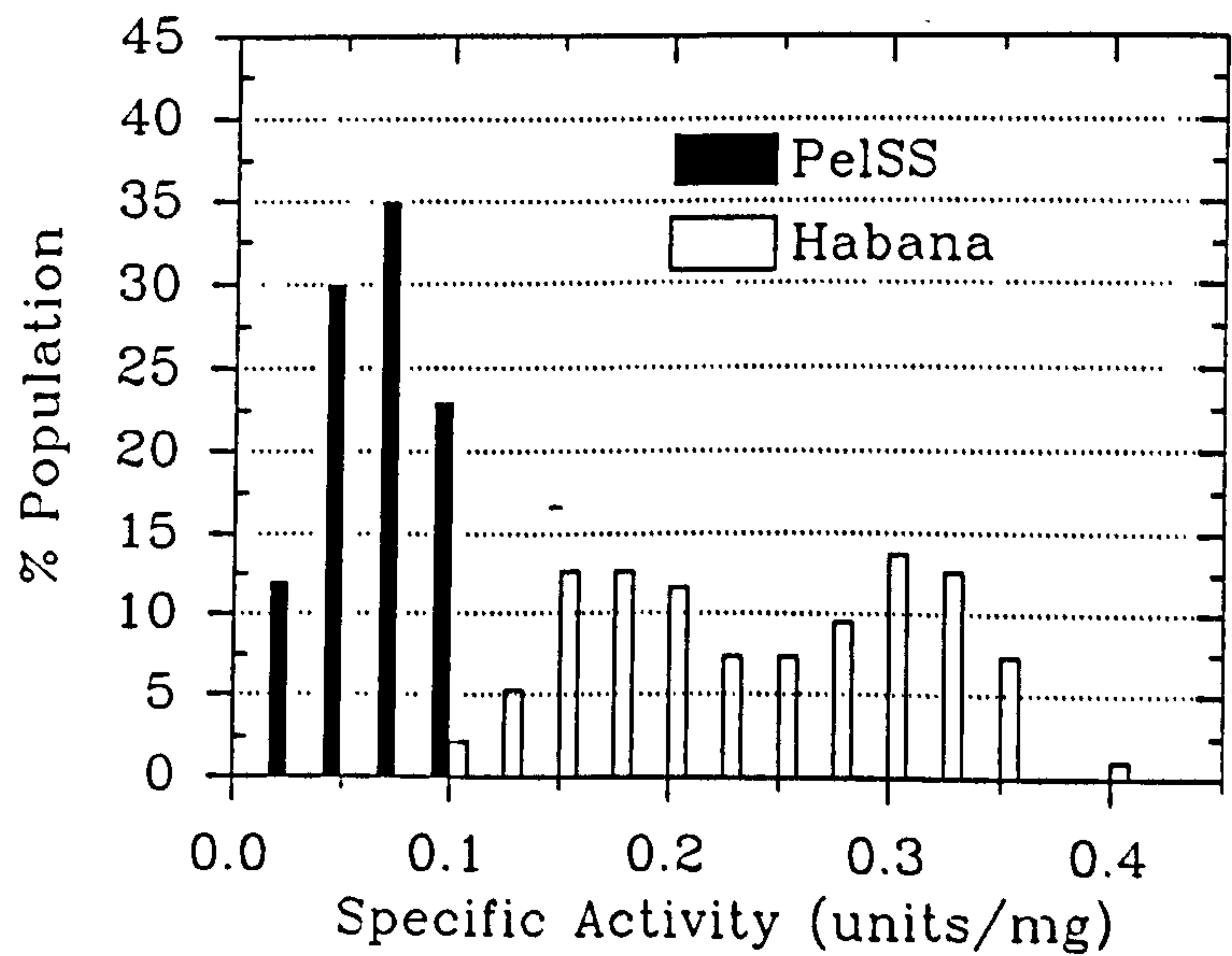
FIGURE 2.4

Esterase activity profiles in fourth instar larvae of the Habana strain with the substrates α -naphthyl acetate (A) and β -naphthyl acetate (B). Data for the standard susceptible PelSS strain are presented for comparison. A unit is equivalent to the hydrolysis of 1 nmole of naphthyl acetate per minute.

A.



B.



The biochemical assay of the Habana strain for altered insensitive AChE, as revealed by the enzymes inhibition profile (FIGURE 2.5 A), showed a high frequency of this mechanism. Again, using the Hardy-Weinberg equation, the frequency of the mechanism in the population was calculated to be 0.85. This is the highest frequency of any field-population yet sampled in the Havana area and suggests that spraying of organophosphates in this area must have been extremely intensive prior to 1986. It is possible also that the spraying of malathion continued in Habana Vieja, the quarter from which the Habana strain originated, until 1990 (J.A. Bisset, personal communication). The highest frequency for the altered acetylcholinesterase mechanism prior to this was 0.24 for the QUIBU population, although a laboratory strain kept under strong selection pressure with malathion showed an increase in frequency for this mechanism to 1.0 (Bisset *et al.*, 1990).

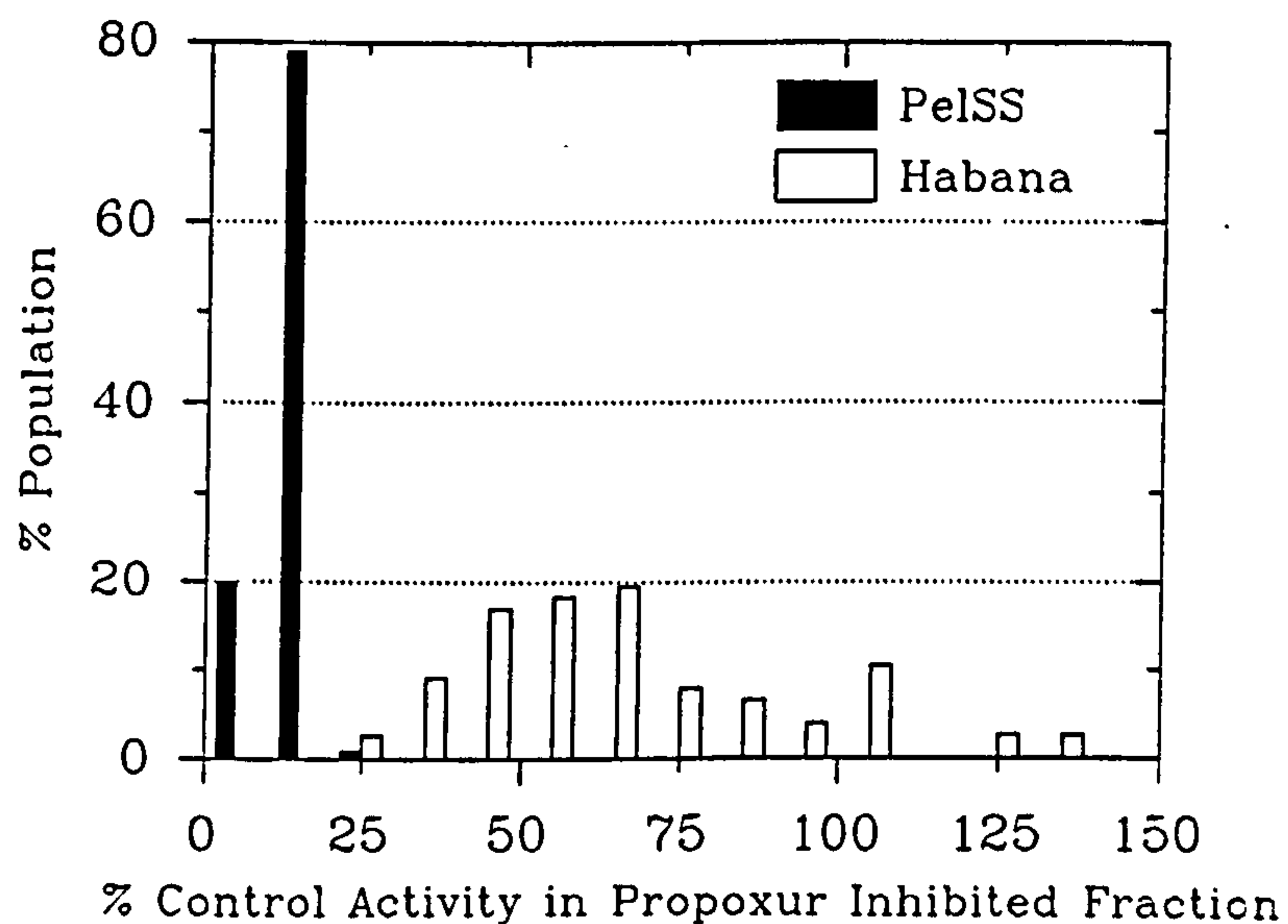
Due to operational difficulties, the esterase and altered acetylcholinesterase assays were not performed on the same batch of individuals and it was not therefore possible to analyze the Habana strain to see if there was any linkage disequilibrium between the two resistance mechanisms. However, analysis carried out on populations from the Habana area by Rodriguez *et al.* (1993) showed that there was no evidence of linkage disequilibrium. This suggested that the selection pressure for double resistance was weak. However, the Rodriguez *et al.* (1993) study also showed that the frequency of individuals homozygous susceptible for both mechanisms was only 0.065, showing that, despite the lack of selection pressure, the elevated esterase and altered acetylcholinesterase mechanisms are relatively stable and, therefore, that organophosphate resistance in Cuban *C. quinquefasciatus* remains common.

Acetylcholinesterase inhibition and glutathione *S*-transferase activity was measured in 100 individual fourth instar PelSS larvae and 175 individual fourth instar Habana larvae

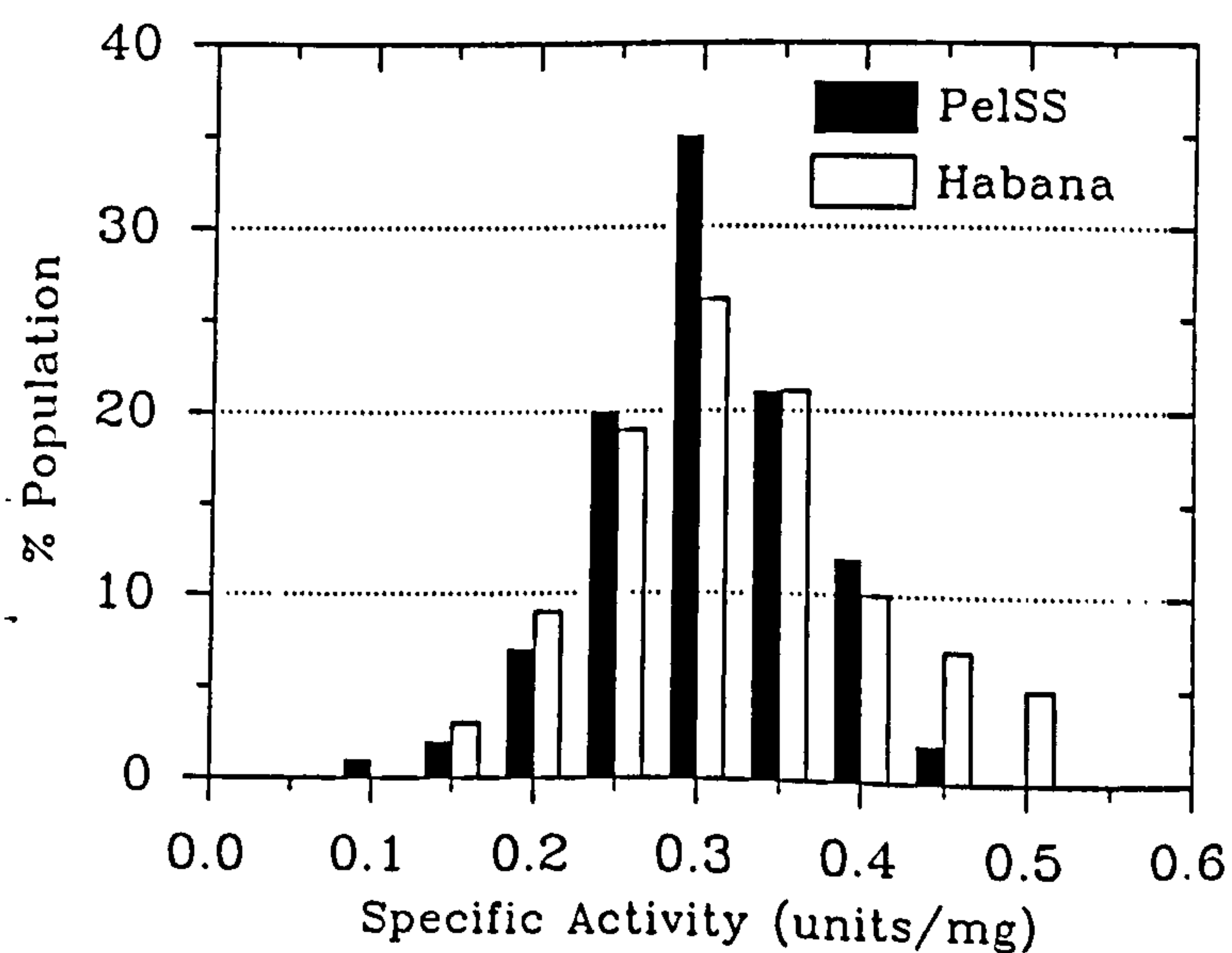
FIGURE 2.5

Acetylcholinesterase inhibition profiles (A) and glutathione *S*-transferase activity profiles (B) in fourth instar larvae of the Habana strain. Data for the standard susceptible PelSS strain are presented for comparison. A unit is equivalent to the production of 1 μ mole of Glutathione-CDNB conjugate.

A.



B.



The profile for glutathione *S*-transferase activity with the general substrate CDNB (FIGURE 2.5 B) showed no differences between the Habana and PelSS strains.

2.3.3. NATIVE POLYACRYLAMIDE GEL ELECTROPHORESIS OF LARVAE

Elevated esterases present in the Habana strain as visualised on native polyacrylamide gels can be seen in FIGURE 2.6. Comparison of the relative mobilities of these esterases with those of previously classified *Culex* esterases (see Villani *et al.*, 1983; Georghiou & Pasteur, 1978; Callaghan, 1989) revealed the presence of esterases Est α 2, Est α 3, Est β 1 and Est β 2, plus a faster migrating esterase α and β which have not, as yet, been classified.

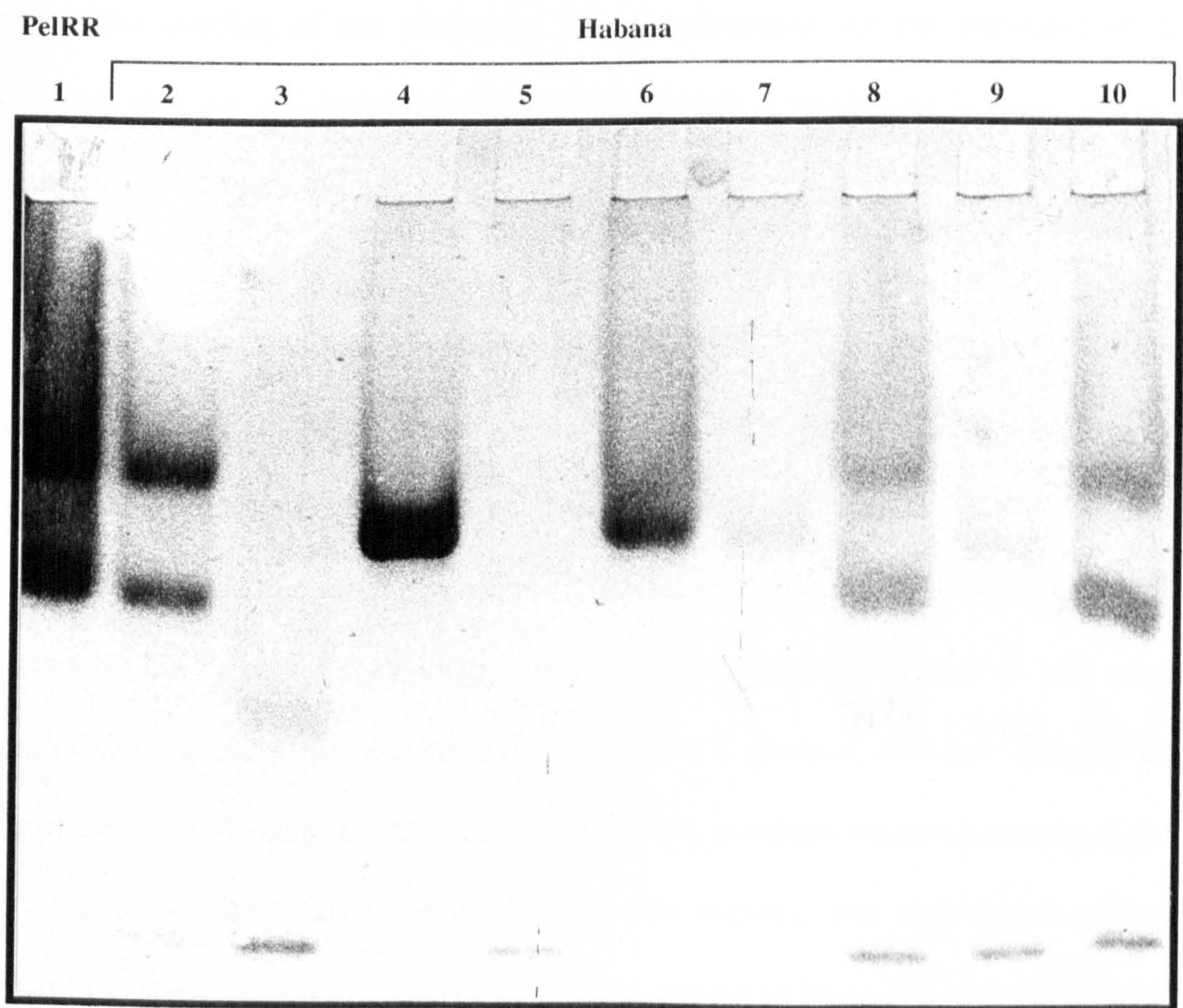
2.3.4. PURIFICATION OF CARBOXYLESTERASE Est β 1

Purification procedures were developed by modifying the methods of Kettermann *et al.*, (1992), Jayawardena (1992) and Karunaratne *et al.* (1993). Under the conditions for chromatography on Q-Sepharose in these methodologies, esterase Est β 1 was found to bind inefficiently, slowly eluting in the homogenisation buffer. Experiments carried out under different conditions revealed that the pH of the Bistris propane buffer should not be below pH 7.0 and that the conductivity of the buffer should not exceed 2.0 mS/cm.

Using the four chromatography steps, Q-Sepharose, phenyl-Sepharose, hydroxy apatite and *p*-chloromercuribenzoate agarose, the esterase Est β 1 could be

FIGURE 2.6

Native PAGE of homogenates of individual fourth instar larvae of the Habana strain showing the differences in elevated esterase banding patterns seen in this strain. A homogenate of PelRR larva, having elevated esterases Est α 2¹ and Est β 2¹ was run in Lane 1 for comparison. Elevated esterases seen in individuals of the Habana strain, listed in order of R_f, are: Lane 2, Est α 2, Est β 2 and an Est β of high R_f; Lane 3, an Est α and the Est β of high R_f; Lane 4, Est β 1 and the Est β of high R_f; Lane 5, Est β of high R_f only; Lane 6, Est β 1 and the Est β of high R_f; Lane 7, Est α 3 and the Est β of high R_f; Lane 8, Est α 2, Est β 2 and the Est α and Est β of high R_f; Lane 9, Est α 3 and the Est α and Est β of high R_f; Lane 10, Est α 2, Est β 2 and the Est α and Est β of high R_f.



separated from all contaminating proteins except esterase Est β 2 which co-eluted with esterase Est β 1 throughout. Since it was known from previous experiments that these esterases had different mobilities on native PAGE, it was decided to employ this method on a Prep-Cell 491 to effect the final separation. By varying the percentage acrylamide, the height of the gel and the wattage used, it was found that optimal separation of the two esterases was obtained as described in section 2.2.8.

High concentrations of DTT were necessary to maintain the active form of esterase B₁. Therefore, 15 mM DTT was included in all buffers used during purification with the exception of the *p*-chloromercuribenzoate-agarose step, where DTT would have prevented binding of the esterases. This requirement for the presence of DTT suggests that the preservation of disulphide bonds is important in maintaining the active form of the enzyme.

Chromatography profiles are shown in FIGURES 2.7-2.9. Esterase Est α 3 eluted from the Q-Sepharose whilst washing the column with homogenisation buffer. Three of the major elevated esterases in the Habana strain, Est α 2, Est β 1 and Est β 2, eluted from the ion-exchange column together in the increasing salt gradient^(Fig. 2.7). The esterases Est β 1 and Est β 2 were separated from esterase Est α 2 on the phenyl-Sepharose column, the esterases β eluting in a second, smaller, activity peak^(Fig. 2.8). Esterases Est β 1 and Est β 2 eluted from the *p*-chloromercuribenzoate-agarose column in a broad activity peak (profile not shown). The low concentration of protein in fractions eluting from this column proved to be so low as to be below the sensitivity of the assay. Esterase Est β 1 was separated from esterase Est β 2 by

FIGURE 2.7

Elution profile of Q-Sepharose chromatography for esterases Est α 2, Est β 1 and Est β 2 from the Habana strain.

5 ml fractions of the elution of 0-0.5 M NaCl gradient in 25 mM Bistris propane (pH 7.0) were collected.

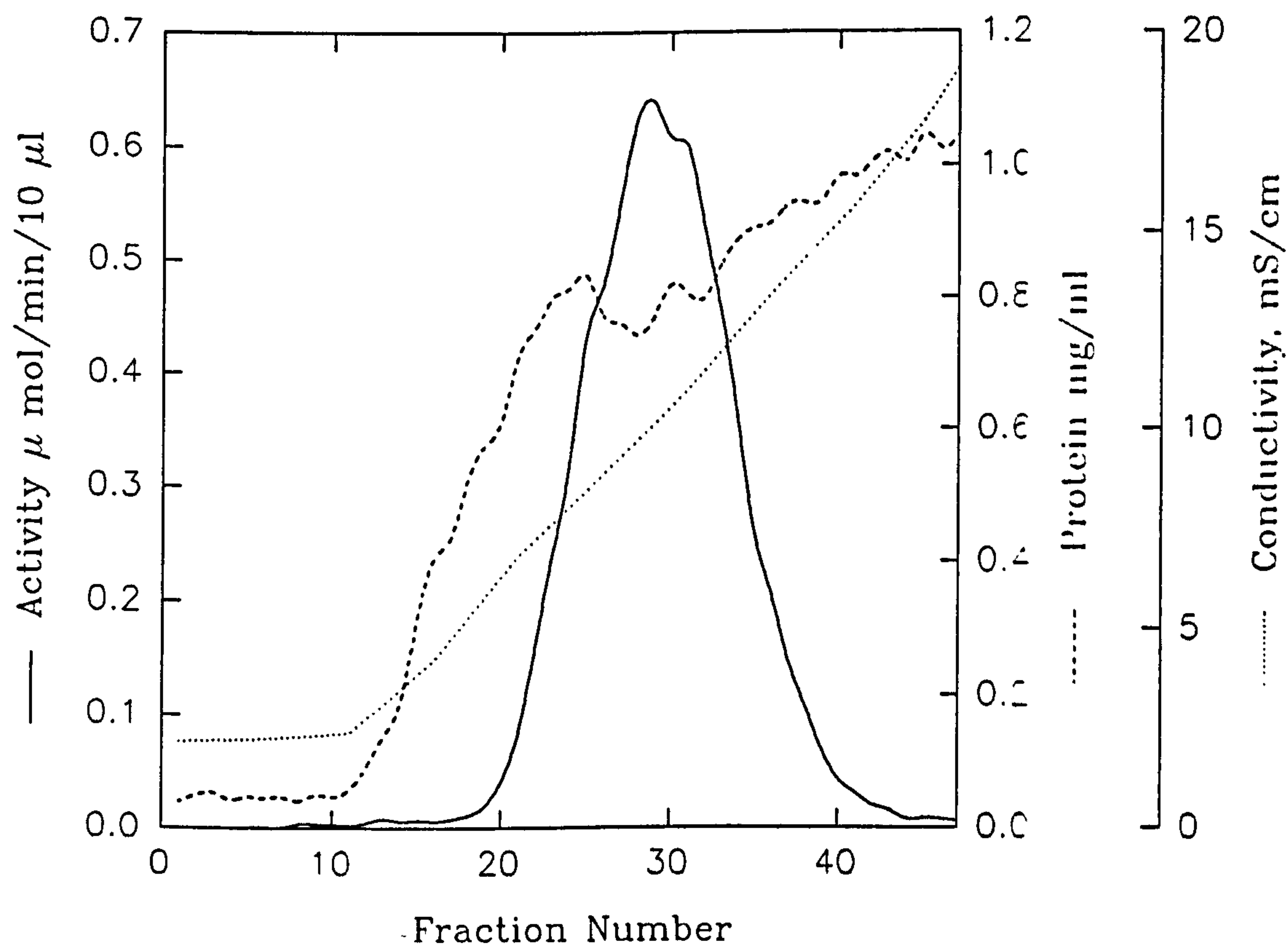


FIGURE 2.8

Elution profile of phenyl-Sepharose chromatography for esterases Est α 2, Est β 1 and Est β 2 from the Habana strain.

5ml fractions of the elution of 3-0 M NaCl gradient in 25 mM Bistris propane buffer (pH 6.5), followed by isocratic elution with the same buffer, were collected. Esterase Est α 2 eluted in a separate peak to esterases Est β 1 and Est β 2 which eluted together.

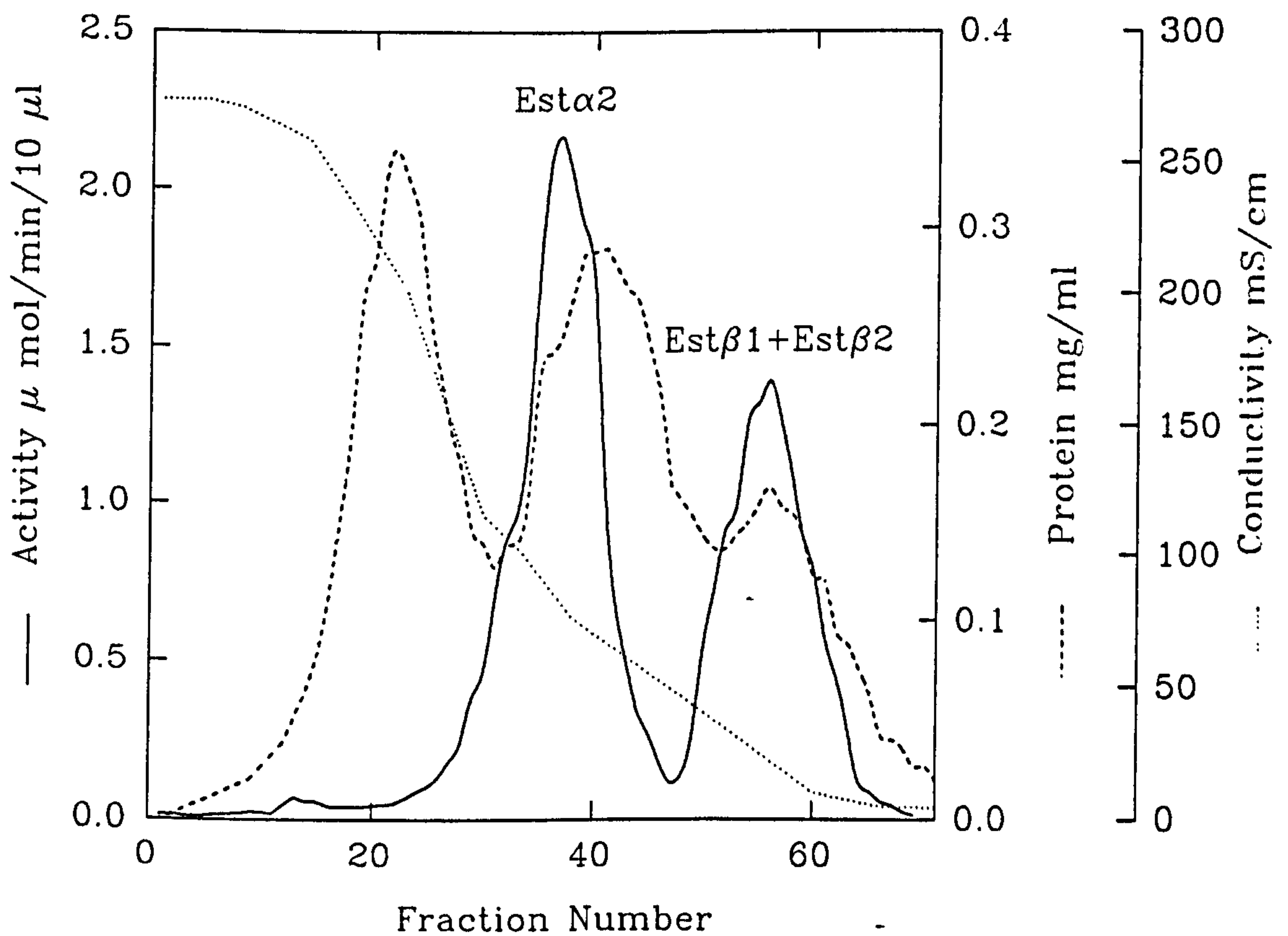
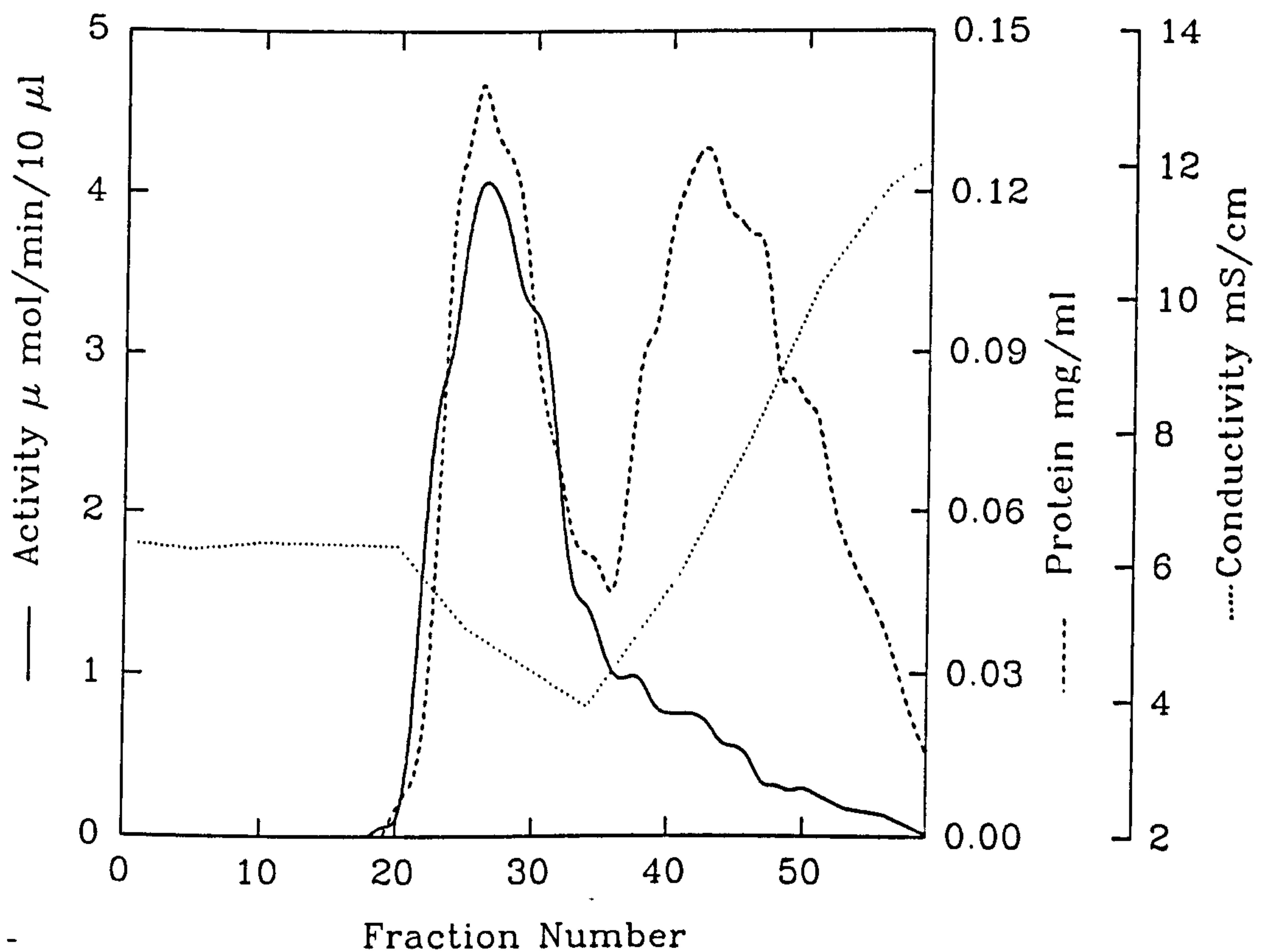


FIGURE 2.9

Elution profile of hydroxyapatite chromatography for esterases Est β 1 and Est β 2 from the Habana strain.

1 ml fractions of the elution were collected. The sample containing the esterases was applied to the column in 10 mM phosphate buffer (pH 6.8) with 50 mM NaCl and eluted with a 10-200 mM phosphate buffer gradient without NaCl.



electrophoresis on a Prep-Cell 491, the esterase Est β 1 eluting as a second activity peak (profile not shown). After completion of the purification procedure, every preparation of esterase Est β 1 was checked for purity on SDS-PAGE. The protein appeared to be homogeneous, only a single band being visible, and any contaminant proteins must have been less than 0.6% of the total protein of the preparation (FIGURE 2.10). Aliquots were run on native PAGE to confirm that the esterase Est β 1 had been completely separated from other esterases. Data for a single representative purification procedure are presented in TABLE 2.2. Data for the purification of Est α 2 and Est β 2 from the same strain are also presented (see Chapter 3.). The activity of the Est β 1 recovered from multiple preparations was approximately 0.3% of the total esterase activity of the crude homogenate and the purification factor was 50-70 fold. The yield of esterase Est β 1 from 15 g wet weight of larvae using the outlined procedure was 50-160 μ g.

2.3.5. CHARACTERIZATION OF ESTERASE *Est* β 1

2.3.5.1. Physical Characterization

The estimated relative monomeric mass of the purified esterase Est β 1 by SDS/PAGE was 71300 ± 1400 kDa (FIGURE 2.11). The M_r of the native enzyme, as estimated from gel filtration chromatography was 66000 ± 7800 kDa (FIGURE 2.12). On gradient 8-25% PAGE the M_r was estimated to be 70300 kDa (FIGURE 2.13), confirming that this esterase is a monomer in its native form. These estimates

FIGURE 2.10

SDS-PAGE of purified esterase Estβ1. Molecular weight markers on 4-20% gel are; Phosphorylase B (106 kDa), bovine serum albumin (80 kDa), ovalbumin (49.5 kDa), carbonic anhydrase (32.5 kDa), soybean trypsin inhibitor (27.5 kDa) and lysozyme (18.5 kDa).

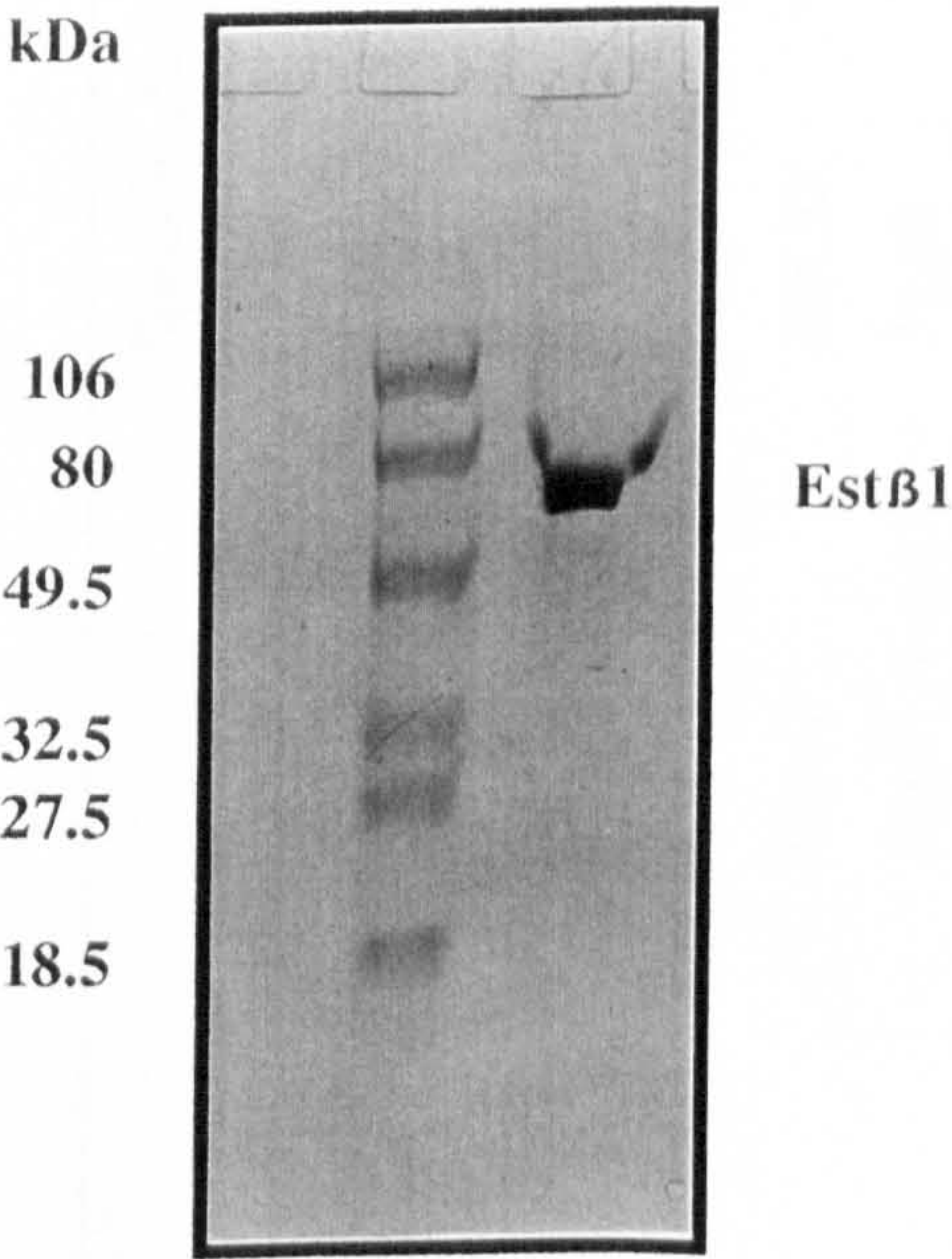


TABLE 2.2. Purification of the esterases Est α 2, Est β 1 and Est β 2

The purification was monitored by the assay of 1 mM *p*-nitrophenyl acetate hydrolysis at 22°C except for the *p*-chloromercuribenzoate-agarose and Prep-Cell steps in which esterase activity was monitored by the assay of 1 mM *p*-nitrophenyl caproate. Specific activities were measured by the assay of 1 mM *p*-nitrophenyl acetate hydrolysis. Est α 2 and the two Est β s were separated after the phenyl-Sepharose column, and Est β 1 and Est β 2 after Prep-Cell electrophoresis.

Step	Specific Activity (Units/mg)	Protein (mg)	Purification (fold)	Recovery (%)
10,000 g supernatant	1.10	773.15	-	-
Q-Sepharose	7.33	100.76	6.66	86.84
phenyl-Sepharose	Est α 2	12.93	27.91	46.67
	Est β 1/Est β 2	14.36	11.99	22.27
Hydroxylapatite	Est α 2	1.02	271.38	35.80
	Est β 1/Est β 2	2.51	36.08	15.84
<i>p</i> -chloromercuribenzoate-agarose	Est β 1/Est β 2	0.28	54.16	8.50
Prep-Cell	Est α 2	0.43	539.85	30.02
	Est β 1	0.09	62.24	0.32
	Est β 2	0.21	58.79	1.40

FIGURE 2.11

Subunit molecular weight estimation of purified esterases Est β 1. R_f values of the SDS-PAGE standard proteins were plotted against their molecular weights (on a log scale). Molecular weight of Est β 1 was determined by linear regression for each gel.

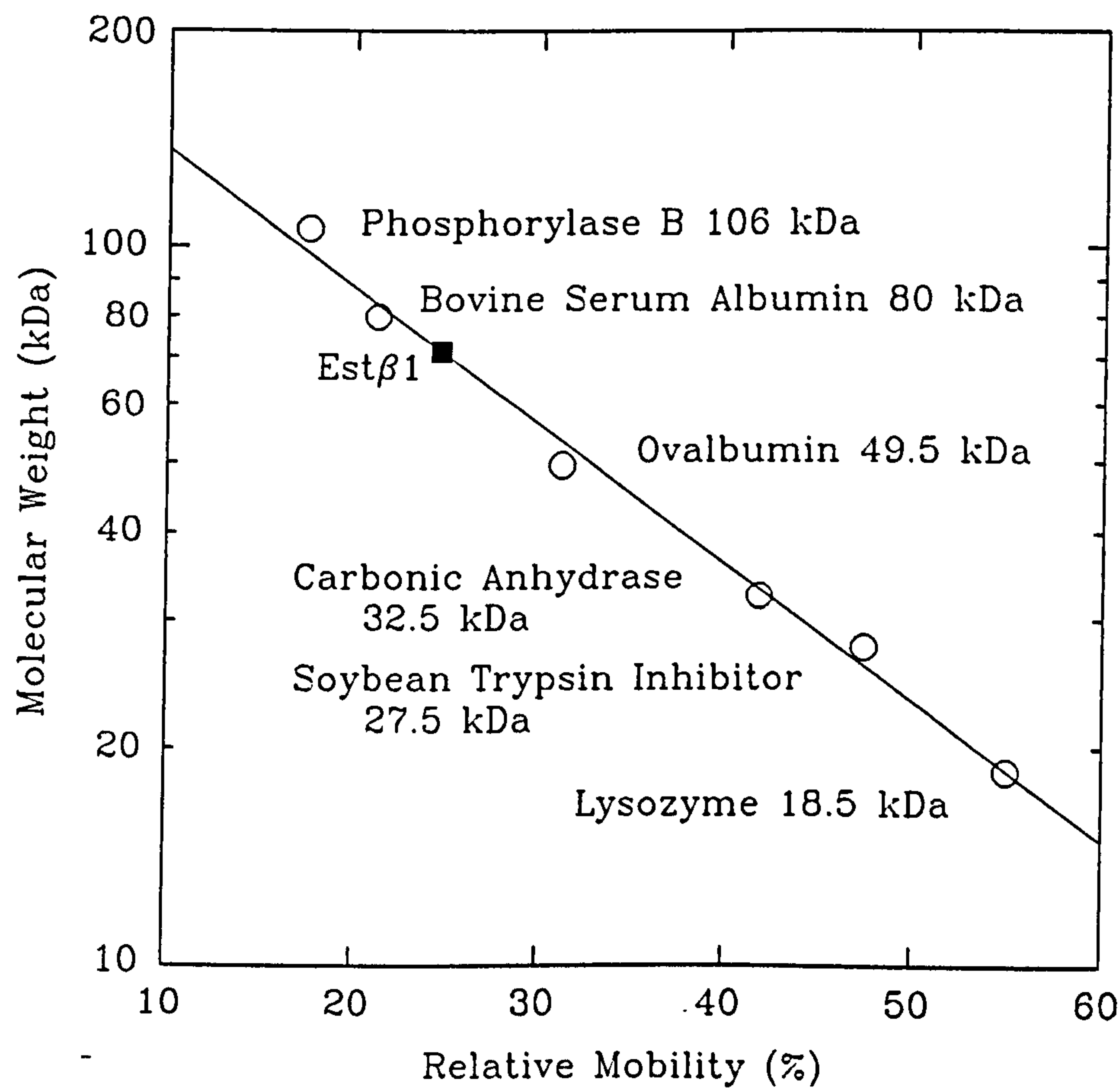


FIGURE 2.12

Native M_r estimation of Est β 1 by FPLC gel filtration chromatography on Superdex 200. The volume of elution (V_e) divided by the void volume (V_o) for standard proteins was plotted against the molecular weights (on a log scale). Molecular weight of esterases Est β 1 was determined by linear regression for each column run.

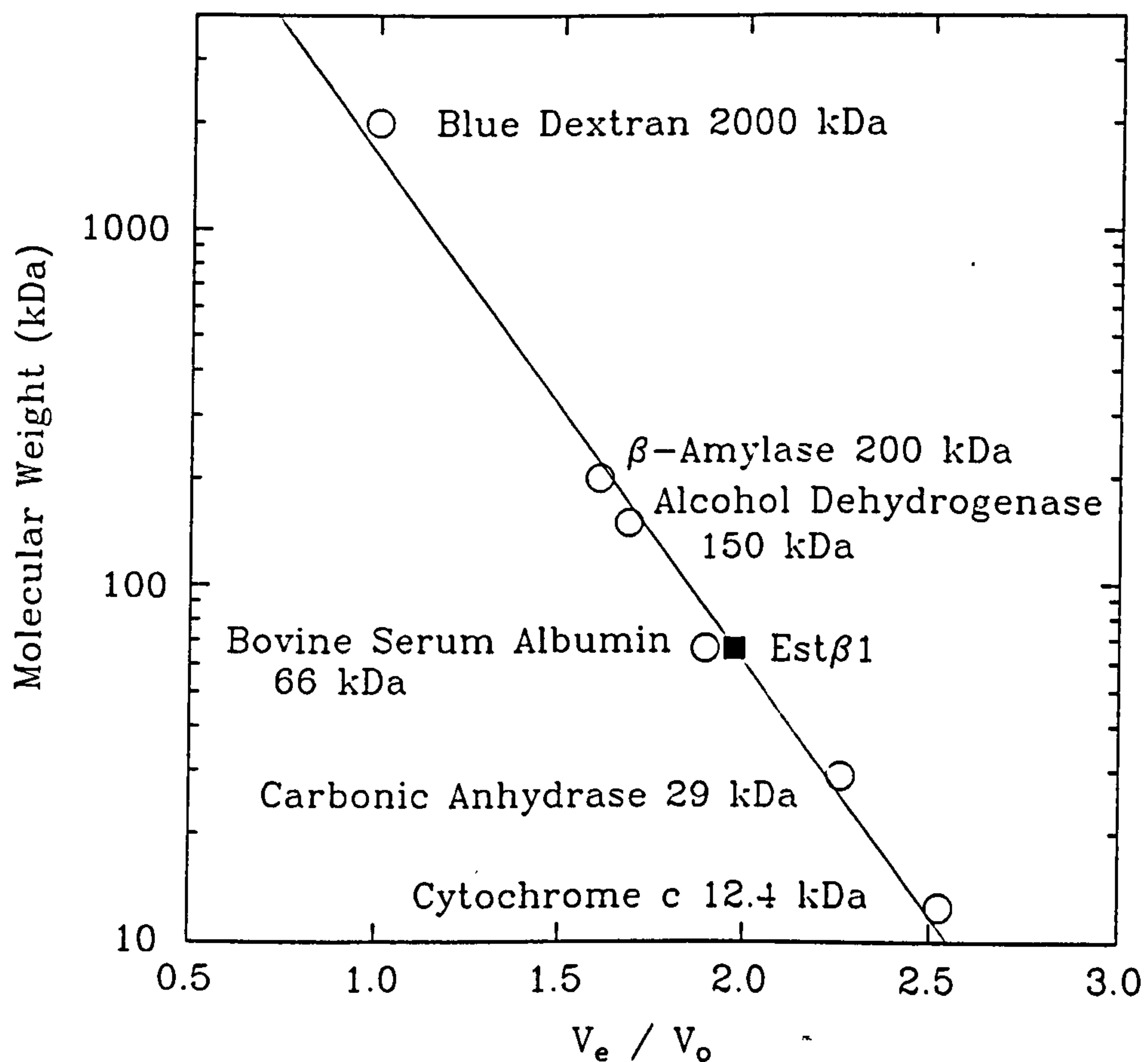
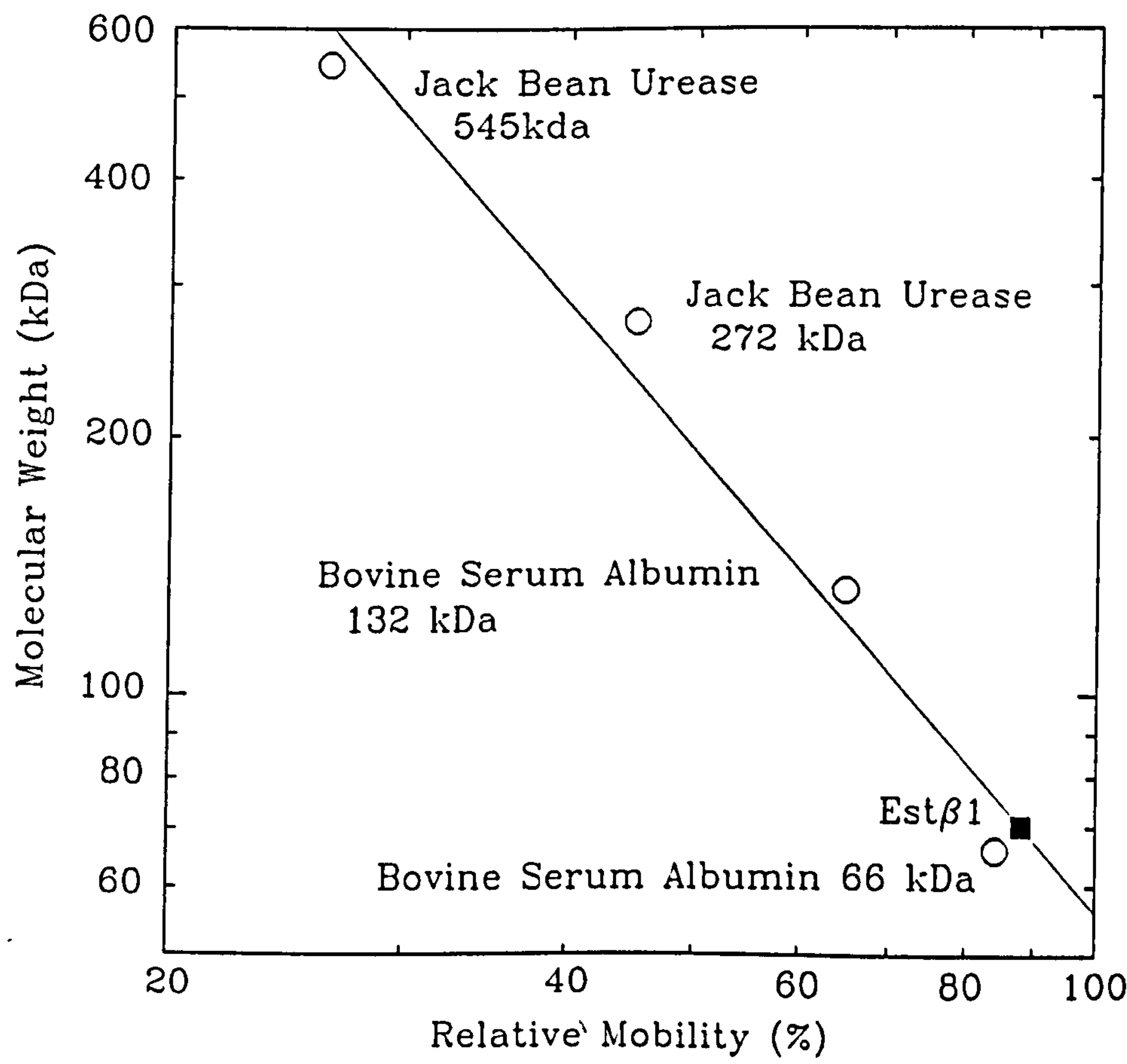


FIGURE 2.13

Native M_r estimation of Est β 1 by native PAGE on 8-25% gradient gels. R_f values of the native PAGE standard proteins were plotted against their molecular weights (both on log scales). Molecular weight of esterase Est β 1 was determined by linear regression.



are in close agreement with those obtained for the independently amplified Est β 1¹ from a Californian strain of *C. quinquefasciatus*, with a reported M_r , under both non-denaturing and denaturing conditions, of 67000 (Fournier *et al.*, 1987), for Est α 2¹ (M_r 67000) (Ketterman *et al.*, 1992) and for Est β 2¹ (M_r 60000) (Karunaratne *et al.*, 1993). They are also in agreement with the size of the Est β 1¹ from the Californian strain calculated from the deduced amino acid sequence of est β 1¹ (M_r 59000) (Mouches *et al.*, 1990). Estimated molecular weights for other insect esterases also fall into this range; esterases E4 and FE4 in the aphid *Myzus persicae* (Devonshire *et al.*, 1986b), esterase-C in the fruitfly *D. melanogaster* (Holwerda & Morton, 1983) and esterases E₁, E₂ and E₃ in the brown planthopper *N. lugens* (Chen & Sun, 1994) all have an M_r of between 62000 and 66000. The pI of 4.8 ± 0.1 for Est β 1, determined by isoelectric focusing on PhastGel IEF (FIGURE 2.14) was slightly lower than those of Est α 2¹ (pI 5.2) (Ketterman *et al.*, 1992) and Est β 2¹ (pI 5.0) (Karunaratne *et al.*, 1993). However, all these pIs are within the range of pH 4.7-6.5 typical of carboxylesterases (Heymann, 1980).

2.3.5.2. Chemical Characterization

2.3.5.2.1. Influence of Effectors

The influence of several metal ions, including a series of transition metal ions, together with some known carboxylesterase effectors, are presented in TABLE 2.3. In contrast to both Est α 2¹ and Est β 2 (Ketterman *et al.*, 1992; Karunaratne *et al.*, 1993), Est β 1 was inhibited by 1 mM concentrations of all metal ions tested.

FIGURE 2.14

Estimation of the pI of esterase Est β 1 by isoelectric focusing on a gel with a gradient of pH 3-9. The distance of IEF standard proteins from the cathodic end of the gel were plotted against their pIs. The pI of Est β 1 was determined by linear regression for each IEF 3-9 and 4-6.5 gel.

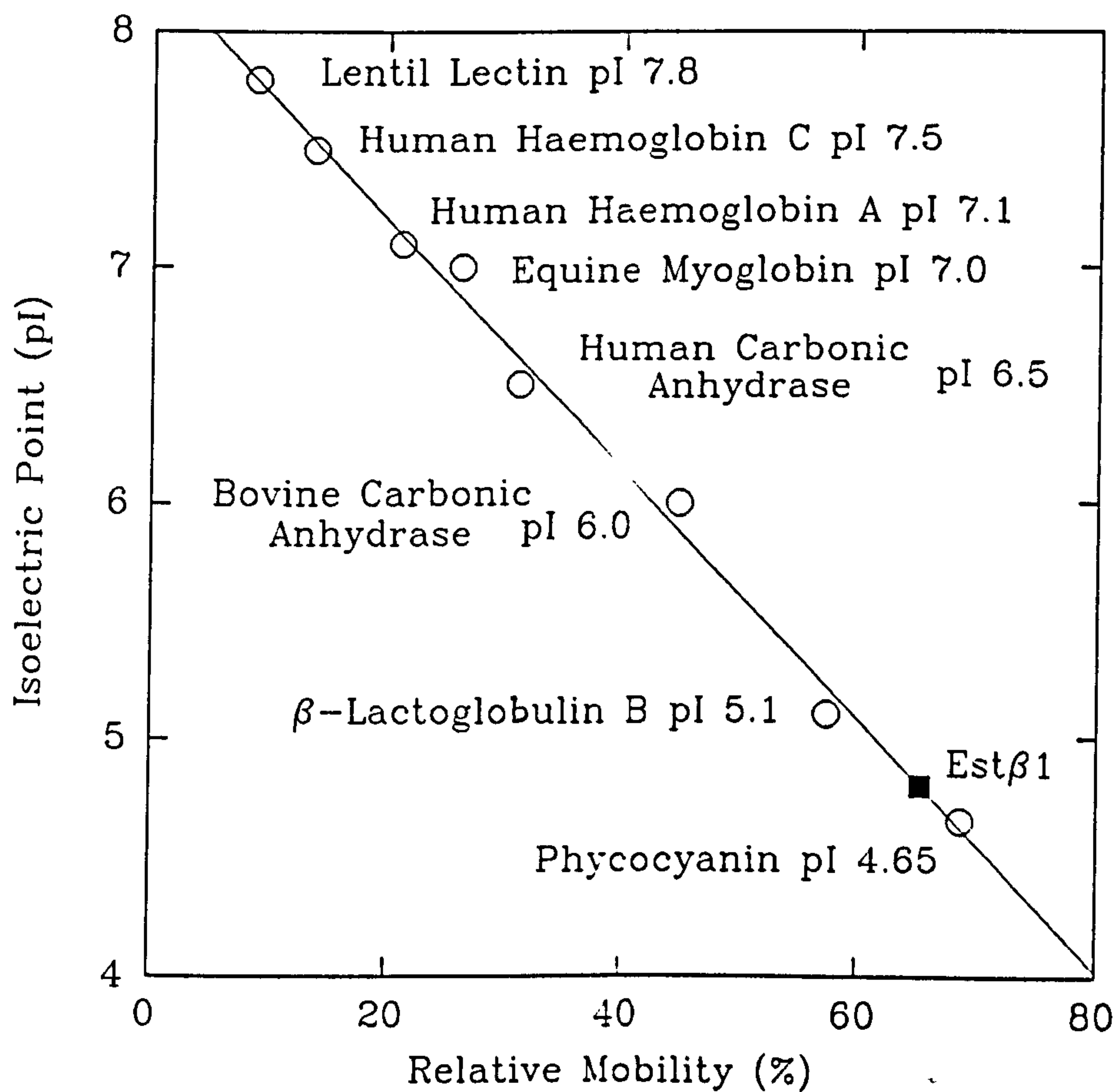


TABLE 2.3. Influence of effectors on the activity of purified EstB1

The effectors were individually pre-incubated with the purified enzyme for 30 min at 22 °C. Esterase activity was then measured with the substrate 1 mM *p*-nitrophenyl acetate in the presence of the relevant effector. The data are means \pm standard deviations.

Effector	Concentration	% Control Activity
MgCl ₂	1.0 mM	26.3 \pm 3.9
CaCl ₂	1.0 mM	26.7 \pm 3.0
MnCl ₂	1.0 mM	30.9 \pm 4.7
FeCl ₂	1.0 mM	18.9 \pm 2.2
CoCl ₂	1.0 mM	20.8 \pm 2.2
NiCl ₂	1.0 mM	21.6 \pm 1.3
CuCl ₂	1.0 mM	37.4 \pm 2.1
ZnCl ₂	1.0 mM	12.8 \pm 1.4
CdCl ₂	1.0 mM	3.3 \pm 0.4
HgCl ₂	1.0 mM	0.2 \pm 0.1
EDTA	1.0 mM	24.0 \pm 2.0
Bis- (<i>p</i> -nitrophenyl) phosphate	0.1 mM	1.1 \pm 0.1
Paraoxon	0.1 μ M	13.4 \pm 0.9
	0.1 mM	1.1 \pm 0.1

Inhibition of EstB1 by Mn^{2+} was significantly higher, and by Cu^{2+} significantly lower, than for EstB2¹ (Karunaratne *et al.*, 1993). As with Est α 2¹ (Kettermann *et al.*, 1992) and EstB2¹ (Karunaratne *et al.*, 1993), EstB1 was inhibited by Hg^{2+} . Previously, inhibition by Hg^{2+} and other transition metal ions has been reported as a characteristic of 'A' esterases [according to Aldridge's (1953a; 1953b) classification] and arylesterases, the metals interacting with the -SH groups of the cysteine residue at the active site (Aldridge, 1993; McCracken *et al.*, 1993). For example, a phosphoric triester hydrolase (an 'A' esterase) purified from the tobacco budworm *H. virescens*, was completely inhibited by Hg^{2+} and slightly inhibited by Cu^{2+} (Konno *et al.*, 1990). However, it is known from their predicted amino acid sequences that both EstB1 and EstB2 are 'B'-type serine hydrolases (Vaughan *et al.*, 1995). Therefore, the Hg^{2+} inhibition of EstB1 and EstB2¹ suggests interaction with a thiol group involved either in catalysis, or in the conformational stability of the enzymes. Other esterases have also shown sensitivity to the presence of metal ions; a carboxylesterase from the cotton aphid *Aphis gossypi* was inhibited by Hg^{2+} , Cu^{2+} , and Cd^{2+} (Owusu *et al.*, 1994), whilst a rat liver microsomal esterases were found to be inhibited to various degrees by Mg^{2+} , Zn^{2+} , Cu^{2+} and Hg^{2+} (Mentlein *et al.*, 1984).

The inhibition of EstB1 by paraoxon shows that under the classification of Aldridge (1953a; 1953b; 1993), it is a 'B'-type esterases. Partially purified TEM-R EstB1¹ was also inhibited by paraoxon (Cuany *et al.*, 1993). EstB1 was inhibited by bis-(*p*-nitrophenyl) phosphate which has been shown to be a specific inhibitor of rat carboxylesterases (Brandt *et al.*, 1980). A similar effect for paraoxon and bis-(*p*-

nitrophenyl) phosphate was found with esterase Est β 2¹ (Karunaratne *et al.*, 1993). Inhibition by the chelating agent EDTA, implying the involvement of a metal ion in catalysis. This is in contrast to Est β 2¹ which was unaffected by EDTA (Karunaratne *et al.*, 1993). Inhibition by EDTA is a characteristic of 'A'-type esterases (Aldridge & Reiner, 1972). Therefore, although Est β 1 is undoubtedly a 'B'-type esterase, it has some properties in common with both groups.

2.3.5.2.2 Substrate specificity

The Michaelis constant (K_m) and V_{max} , determined for the hydrolysis of *p*-nitrophenyl acetate and *p*-nitrophenyl caproate by esterase Est β 1 are presented in TABLE 2.4. Data for Est α 2¹ and Est β 2¹ (Karunaratne *et al.*, 1993) are presented for comparison. As with these esterases, Est β 1 showed a higher affinity towards *p*-nitrophenyl caproate (C_6) than *p*-nitrophenyl acetate (C_2). A preference for medium length mono- and di-acylglycerols has been observed previously for both esterases Est α 2¹ and Est β 2¹ (Jayawardena, 1992; Kettermann *et al.*, 1992). The K_m and V_{max} of Est β 1 are similar to those of Est β 2¹. The rates of hydrolysis of both substrates by the esterases β are lower than for Est α 2¹. The K_m of the non-elevated Est β 1³, purified from the susceptible PelSS strain, with *p*-nitrophenyl acetate was higher than that of the Habana Est β 1 (Karunaratne, 1994), thus showing that these two electrophoretically identical esterases differ in their substrate specificities. Esterases E₁, E₂ and E₃ purified from the insect *N. lugens* had Michaelis constants and V_{max} s for the substrate *p*-nitrophenyl acetate similar to those for the esterases B, with K_m

TABLE 2.4. Substrate interactions of purified Habana carboxylesterase Estβ1

The rate of hydrolysis of both substrates used was measured at 405 nm. A unit corresponds to the hydrolysis of 1 μmol of substrate in 1 min under the assay conditions stated. The Michaelis constants for the PeI_{RR} Estα2¹ and Estβ2¹ are presented for comparison (Karunaratne *et al.*, 1993). The data are means ± standard deviations.

Esterase	p-nitrophenyl acetate		p-nitrophenyl caproate	
	Parameter...	V_{\max} (units/mg)	K_m (μM)	V_{\max} (units/mg) K_m (μM)
Habana Estβ1		84.3 ± 3.0	109.2 ± 10.9	97.1 ± 5.9 7.9 ± 2.5
PeI _{RR} Estα2 ¹		472.0 ± 51.4	145.8 ± 45.0	788.2 ± 74.8 35.4 ± 9.7
PeI _{RR} Estβ2 ¹		63.4 ± 0.8	140.0 ± 50.0	83.3 ± 9.8 17.0 ± 6.0

V_{\max} and K_m values for Habana Estβ1 were calculated from three replicates, each replicate using enzyme from a different purification

values from 150-250 μM and V_{max} values from 20-100 unit/mg (Chen & Sun, 1994), whilst pig and rat carboxylesterase isoenzymes have values for the same constants of 30-220 μM and 66-110 units/mg respectively (Heymann, 1980). These observations are in close agreement with those for Est β 1.

2.3.5.2.3. *Interaction of Est β 1 with insecticides*

The second-order rate constants for the formation of the acylated enzyme (bimolecular rate constant), k_a , for the interaction of Est β 1 with four organophosphates (malaoxon, fenitrooxon, chlorpyrifos-oxon and paraoxon) and one carbamate (propoxur) are shown in Table 2.5. Values of k_a for Est α 2¹ and Est β 2¹ purified from the Sri Lankan PelRR strain (Karunaratne *et al.*, 1993) are presented for comparison. The potency of chlorpyrifos-oxon and paraoxon as inhibitors was so high that the k_a could only be determined in the presence of substrate (Main & Dauterman, 1963).

The pyrethroid insecticide lambda cyhalothrin had no effect on activity of the esterase B₁ even at its solubility limit showing that this esterase is not involved in the resistance of the Habana population to pyrethroids. In contrast, the organophosphate sequestering carboxylesterase E4 from *Myzus persicae* is able to hydrolyse the pyrethroid permethrin (Devonshire & Moores, 1982) and interacts reversibly with other pyrethroids (Devonshire & Moores, 1989) thereby causing resistance to these insecticides.

TABLE 2.5. The kinetic constant k_a for insecticide interactions with purified Habana esterase Estβ1

Enzyme activity was measured by the assay of 1 mM *p*-nitrophenyl acetate hydrolysis at 22 °C. The k_a values for the PelRR Estα2¹ and Estβ2¹ are presented for comparison (Karunaratne *et al.*, 1993). The data are means ± standard deviations.

Insecticide	10 ⁻⁵ x k_a (M ⁻¹ min ⁻¹)		
	Habana Estβ1	PelRR Estα2 ¹	PelRR Estβ2 ¹
Malaoxon [*]	0.16 ± 0.02 n=43	0.17 ± 0.07	0.50 ± 0.17
Fenitrooxon [*]	1.52 ± 0.32 n=21	0.91 ± 0.37	1.73 ± 0.60
Chlorpyrifos-oxon [*]	1340 ± 88 n=8	145.3 ± 59.7	1550 ± 140
Paraoxon [*]	161 ± 35.7 n=12	178 ± 47.7	170 ± 53
Propoxur [†]	0.0017 ± 0.0002 n=29	0.012 ± 0.003	0.0052 ± 0.0017
[*] Organophosphate			
[†] Carbamate			

To test whether the difference between two mean values was statistically significant at the $P=0.05$ level or higher, the standard deviations of the means were multiplied by 1.97 and added or subtracted from the means themselves. Where this gave values that did not overlap, the means were adjudged to be statistically significantly different from each other.

It has been shown previously that the bimolecular rate constant, k_a , correlates most closely with the observed resistance (Karunaratne *et al.*, 1993; Hemingway *et al.*, 1993). Amplification of *estβ1* is less common than co-amplification of *estα2* and *estβ2*. It was thought that Estβ1 might have a lower affinity for organophosphates than Estα2 and Estβ2, leading to lower resistance and a lower selective advantage of this phenotype in the presence of these insecticides. However, the k_a s with a range of OPs for purified Estβ1 were not significantly different to those of Estβ2¹, and were higher with some OPs than Estα2. The relative efficacy of the insecticide binding of these esterases, therefore, can not explain why amplification of *estβ1* is less common than esterases *estα2* and *estβ2*, or why *estα2* and *estβ2* have been able to apparently out compete *estβ1* in field populations of *C. quinquefasciatus* in California (Raymond *et al.*, 1987) and Cuba.

The k_a for Estβ1³, purified from the susceptible PelSS strain of *C. quinquefasciatus*, with paraoxon was only $1.94 \times 10^5 \text{ M}^{-1} \text{ min}^{-1}$ (Karunaratne *et al.*, 1995a), nearly 100-fold less than for Estβ1 and Estβ2¹. This shows that the elevated 'resistant' esterases β have a greater ability to bind the insecticide than their non-elevated 'susceptible' counterparts. Amplified carboxylesterase E4 from the insect *Myzus persicae* also had a high k_a ($1330 \times 10^5 \text{ M}^{-1} \text{ min}^{-1}$) for paraoxon (Devonshire, 1977). The value of this constant with paraoxon for AChE, the target of organophosphates, purified from the plant bug *Lygus hesperus* was only $9.44 \times 10^5 \text{ M}^{-1} \text{ min}^{-1}$ (Zhu & Brindley, 1990) and that of a susceptible AChE from *H. virescens* was only $0.3 \times 10^5 \text{ M}^{-1} \text{ min}^{-1}$ (Brown & Bryson, 1992). This demonstrates the protective value of the amplified esterases which, by virtue of their higher affinity for the

organophosphates, can sequester the insecticide before it can reach its target site. The AChE in some OP resistant strains has been shown to be altered, the altered form having a lower affinity for OPs and carbamates than the 'susceptible' form. For example, in an experiment in which sub-colonies of the PelRR strain (which, in addition to having the elevated Est α 2/Est β -based mechanism, also has a low frequency of altered AChE) was selected with chlorpyrifos, malathion and propoxur, the AChE in crude homogenates of the chlorpyrifos-selected subcolony was found to have an inhibition co-efficient (k_i) with propoxur of 5.12×10^3 compared to values of this constant for AChE in homogenates of the malathion and propoxur-selected colonies, and also the PelSS strain, of between $1.02 - 1.83 \times 10^5$. Selection of this mechanism along with the elevated esterase-based mechanism is common in field populations of *C. quinquefasciatus* (Bisset *et al.*, 1990; Bisset *et al.*, 1991; Villani & Hemingway, 1987). the two mechanisms together having a multiplicative effect. A Cuban strain, MRES, homozygous for both the elevated Est β 1 and altered AChE mechanisms was 1050-fold more resistant to the organophosphorus insecticide malathion, and 136-fold more resistant to the carbamate propoxur (Bisset *et al.*, 1990), whilst a Californian strain, TEM-R, homozygous for the amplified *est β 1* mechanism alone, and containing a higher copy number of the *est β 1*² gene than MRES, was only 142-fold resistant to malathion and 1.5-fold resistant to propoxur (Georghiou *et al.*, 1980).

Turning to the vertebrate esterases, a k_a value of $46.0 \pm 7.0 \times 10^5 \text{ M}^{-1} \text{ min}^{-1}$ has been reported for rat plasma carboxylesterases with paraoxon (Maxwell, 1992), this compared with a k_a of $9.0 \pm 1.1 \times 10^5 \text{ M}^{-1} \text{ min}^{-1}$ for rat brain AChE with the same

insecticide (Maxwell, 1992). The k_a of AChE in sheep erythrocytes with paraoxon was similar to that of the rat brain AChE, being $11 \times 10^5 \text{ M}^{-1} \text{ min}^{-1}$ (Aldridge & Davison, 1952). *In vivo* toxicology studies with rats have shown that, as with *C. quinquefasciatus* esterases, rat carboxylesterases are more sensitive to organophosphates than AChE and, therefore, can protect AChE from inhibition by these insecticides (Gupta & Dettbarn, 1993). These data show that, as with the elevated insect esterases mentioned above, vertebrate carboxylesterases can, by virtue of their higher reactivity with organophosphate insecticides, protect the target site, AChE, by binding them before they can reach the AChE. The rat carboxylesterase, like the *C. quinquefasciatus* Est α 2, Est β 2 (Hemingway *et al.*, 1993), and Est β 1 (in this study), show an inverse relationship between their *in vivo* protection and their relative *in vitro* reactivity with OPs (Maxwell, 1992). This suggests that in both animals, esterase detoxification gives the greatest protection with the highly toxic OPs (ie. those OPs with the highest reactivities for AChE and the lowest LD50 values) when compared to the less toxic OPs.

2.4. SUMMARY

Combined bioassay, synergist and biochemical assay data suggest that heavy use of malathion for 7 years up to 1986 had selected for two resistance mechanisms, elevated esterases and altered insensitive AChE, in the Habana *C. quinquefasciatus* population. Despite reduction in the use of malathion since 1986, these mechanisms are still present at high frequencies in the population. The two mechanisms give cross-resistance to a broad range of OPs and carbamates, the exception being with

pirimiphos-methyl, to which there was little resistance, even in the MRES strain which is homogeneous for both mechanisms (Bisset *et al.*, 1991). Native PAGE of crude homogenates of fourth instar larvae from this strain revealed the presence of four major elevated esterases; Est α 2, Est α 3, Est β 1 and Est β 2. Using four sequential chromatography steps and an electrophoresis step on a Prep-Cell, esterase B₁ was purified to homogeneity. Apart for the differences in their interaction with metal ions and EDTA, the biochemical characteristics of Est β 1 and Est β 2¹ were remarkably similar. This, together with their high degree (~98%) of homology at both the nucleotide and predicted amino acid levels (Vaughan *et al.*, 1995), suggests that they were originally alleles of the same locus. The elevated 'resistant' Est β 1 is kinetically distinct from the 'susceptible' Est β 1³ purified from the PelSS strain (Karunaratne *et al.*, 1995a). These esterase also differ at the nucleotide level (Vaughan *et al.*, 1995). This suggests that insecticide resistance is therefore conferred through amplification of alleles coding for esterases which have a greater specificity for the insecticides they sequester than their non-amplified counterparts.

Evidence from the insecticide interactions of Habana Est β 1, PelRR Est α 2¹ and Est β 2¹ (Karunaratne *et al.*, 1993), suggests that the hypothesis that the co-elevated Est α 2 and Est β 2 might have spread into *C. quinquefasciatus* populations in Cuba by virtue of a higher efficacy for the binding of organophosphorus insecticides than the Est β 1 is incorrect. Indeed, the esterase B₁ had a higher affinity for some insecticides than Est α 2¹ and, as mentioned above, had affinities very similar to Est β 2¹.

CHAPTER 3

Chapter 3

PURIFICATION AND CHARACTERIZATION OF CARBOXYLESTERASES

Est α 2 AND Est β 2 FROM THE HABANA STRAIN

3.1. INTRODUCTION

The co-amplified esterase genes *est α 2* and *est β 2* have been found in organophosphate resistant *C. quinquefasciatus* populations on at least three continents (Raymond *et al.*, 1991). Restriction maps of the amplified *est β 2* gene and its flanking regions from African, Asian and North American populations were found to be identical whilst those of the non-amplified alleles showed a high level of variability, thus prompting the hypothesis that the *est β 2* gene has been amplified only once and has subsequently spread worldwide by migration (Raymond *et al.*, 1991). If this is so, one would expect the expressed Est β 2 in all *C. quinquefasciatus* strains having this amplified esterase gene to be identical and, therefore, their biochemical characteristics also to be identical. Since Est α 2 has always been found co-amplified with Est β 2 (Raymond *et al.*, 1991) and since the *est α 2¹* gene has been found recently to sit on the same amplicon as the *est β 2¹* gene (Vaughan, 1995), the same should be true of this esterase. Following on from this, since the Est α 2 and Est β 2 should be identical, the strains carrying them (and no other OP resistance mechanisms) also should have similar cross-resistance spectra to organophosphorus insecticides. The observation that *C. quinquefasciatus* strains from different geographical areas which have only the elevated esterase Est α 2/Est β 2-based

mechanism of organophosphorus insecticide resistance actually have different cross-resistance spectra (Georghiou & Pasteur, 1978; Amin & Peiris, 1990; Hemingway *et al.*, 1990; Peiris & Hemingway, 1990a; Peiris & Hemingway, 1990b) suggested this hypothesis might be incorrect. In addition, it has been found that elevated esterases Est α 2 and Est β 2 purified from strains of different geographic origin, and from sub-colonies of a single strain (Sri Lankan PelRR) selected with different insecticides, showed variation in their bimolecular rate constants (k_a s) with various insecticides tested and it was suggested that these esterases may either be different allelic forms of a single Est α 2 and Est β 2, or mixtures of different allelic forms of these esterases (Karunaratne *et al.*, 1993; Jayawardena *et al.*, 1994; Ketterman *et al.*, 1993; Karunaratne, 1994). To explain this result and also the variation in cross-resistance spectra, it was hypothesised that the *est α 2* and *est β 2* genes might have been co-amplified several times, different alleles of these genes being amplified at each occurrence (Ketterman *et al.*, 1993).

More recently, data have been reported supporting the former (Raymond *et al.*, 1991) hypothesis. Southern blots of PelRR and PelSS *Eco*R1 genomic digests were probed with cDNA fragments of the PelRR *est α 2*¹ and *est β 2*¹ esterase genes (Karunaratne *et al.*, 1995a). The restriction fragment of the amplified *est β 2*¹ gene in PelRR was the same size as that reported by Raymond *et al.* for *est β 2* in other strains (Raymond *et al.*, 1991). However, the PelRR strain was also found to contain non-amplified *est α* and *est β* genes. The RFLP band of the non-amplified *est β* was the same size as the *est β 1*³ gene in PelSS, but the non-amplified *est α* was slightly different in size to the *est α 3* gene in PelSS suggesting the presence of a different

estα allele. It is unlikely that the purification methodologies employed would have separated the expressed esterases of these genes from the elevated esterases. Therefore, it was thought possible that the variation in the k_a s of the enzyme preparations from PelRR and other strains having elevated Estα2 and Estβ2 might be due to minor contamination with different non-elevated esterases (Karunaratne *et al.*, 1995a), and/or to different levels of amplification of *estα2* and *estβ2* with concomitant variation in the proportion of non-elevated esterases present. Indeed, whilst only a single non-amplified *estα* and *estβ* allele was detected in the PelSS strain (Karunaratne *et al.*, 1995a), restriction fragment analysis of non-amplified *estα* and *estβ* alleles in field populations have shown a high level of polymorphism (Raymond *et al.*, 1991). Since the PelRR strain still contains non-amplified *estα* and *estβ* alleles, it is possible that other non-amplified alleles might exist in other populations of *C. quinquefasciatus* having co-amplified *estα2* and *estβ2*. This might in turn have led to the observed variations in insecticide cross-resistance spectra.

As mentioned previously in Chapter 2., elevated esterases Estα2 and Estβ2 were first observed in populations of Cuban *C. quinquefasciatus* in 1991, only elevated Estβ1 being observed prior to this. Since their appearance, these two esterases have spread rapidly, prompting the hypothesis that they may have a higher efficacy for binding OPs than Estβ1. Evidence against this hypothesis was found in the observation that purified Habana Estβ1 actually had a higher affinity for some OPs than PelRR Estα2¹, and had affinities very similar to Estβ2¹ (see Chapter 2.). However, two reasons prompted the decision to examine the insecticide kinetics and other characteristics of Habana Estα2 and Estβ2. Firstly, given the relative isolation

of Cuba, in terms of the small number of visiting planes and ships, the chances of these esterases arriving by migration was thought to be slim. Therefore, it was thought possible that their appearance may have been by a novel amplification of *estα2* and *estβ2* genes in Cuban *C. quinquefasciatus*. Secondly, all of the esterases *Estα2* and *Estβ2* so far characterised have been purified from strains originating from Sri Lanka, Tanzania and Saudi Arabia (Karunaratne *et al.*, 1993; Jayawardena *et al.*, 1994; Kettermann *et al.*, 1993; Karunaratne *et al.*, 1995a). This study offered the opportunity of purifying and characterising these esterase from a strain originating from the Americas.

3.2. MATERIALS AND METHODS

The origin of the Habana strain and conditions for mosquito colony maintenance are detailed in sections 2.2.1. and 2.2.2..

The chemicals and equipment required for the purification and characterisation of *Estα2* and *Estβ2* were the same as detailed for *Estβ1* in section 2.2.3.

3.2.1 PURIFICATION AND CHARACTERISATION OF ESTERASES *Estα2* AND *Estβ2*

The method for purification of Habana *Estα2* was a development of that used for this esterases in other strains (Kettermann *et al.*, 1992; Kettermann *et al.*, 1993). The purification was begun by chromatography of a homogenate of fourth instar larvae

of the Habana strain on Q-Sepharose and phenyl-Sepharose Fast Flow columns under the conditions detailed in section 2.2.8. Whilst the conditions for Q-Sepharose chromatography used by Kettermann *et al.* (1992) would have worked equally as well for the purification of Est α 2, the lower pH (pH 6.5) and higher conductivity of the Bistris propane buffer used in this methodology was not suitable for the purification of Est β 1 which was carried out on the same homogenates as used in the present study. The esterase Est α 2 peak was eluted first from the phenyl-Sepharose column, the fractions were combined and dialysed against dry sucrose. Further de-salting and buffer exchange into 10 mM sodium phosphate buffer (pH 6.8), containing 10 mM DTT, was performed on PD-10 columns. The sample was then applied to a hydroxyapatite column equilibrated with the same buffer. After washing the column with 5 column volumes of the start buffer, the esterase activity was eluted with a 5 bed volume gradient of sodium phosphate buffer (10-200 mM, pH 6.8). This step was performed at 22°C to avoid crystallization of sodium phosphate in the buffer. The esterase was eluted as a single peak, the fractions were combined and concentrated down to a volume of 400 μ l in Amicon Centriprep 10 and Centricon 10 concentrator units. Blue marker [see section 2.2.8.] was mixed with the concentrated enzyme in a ratio of 1:5 and the whole applied to a 9 cm high 9% acrylamide gel with a 0.5 cm 4 % acrylamide gel in a 28 mm ID cell of a Prep-Cell model 491. The gel was run at 15 W constant power and the enzyme eluted with the electrode buffer, 0.1 M Tris/borate, pH 8.0, with 2 mM EDTA, 10 mM DTT. Thereafter the enzyme was treated and stored as for Est β 1 (see section 2.2.8.).

Esterase Est β 2 was purified using the same method as for the Est β 1 purification

detailed in section 2.2.8.

Both esterases were obtained from several purifications, and from several generations of mosquitoes for use in subsequent characterizations to ensure that all results were reproducible.

Characterizations of the purified esterases were carried out according to the procedures described in section 2.2.9.

3.3. RESULTS AND DISCUSSION

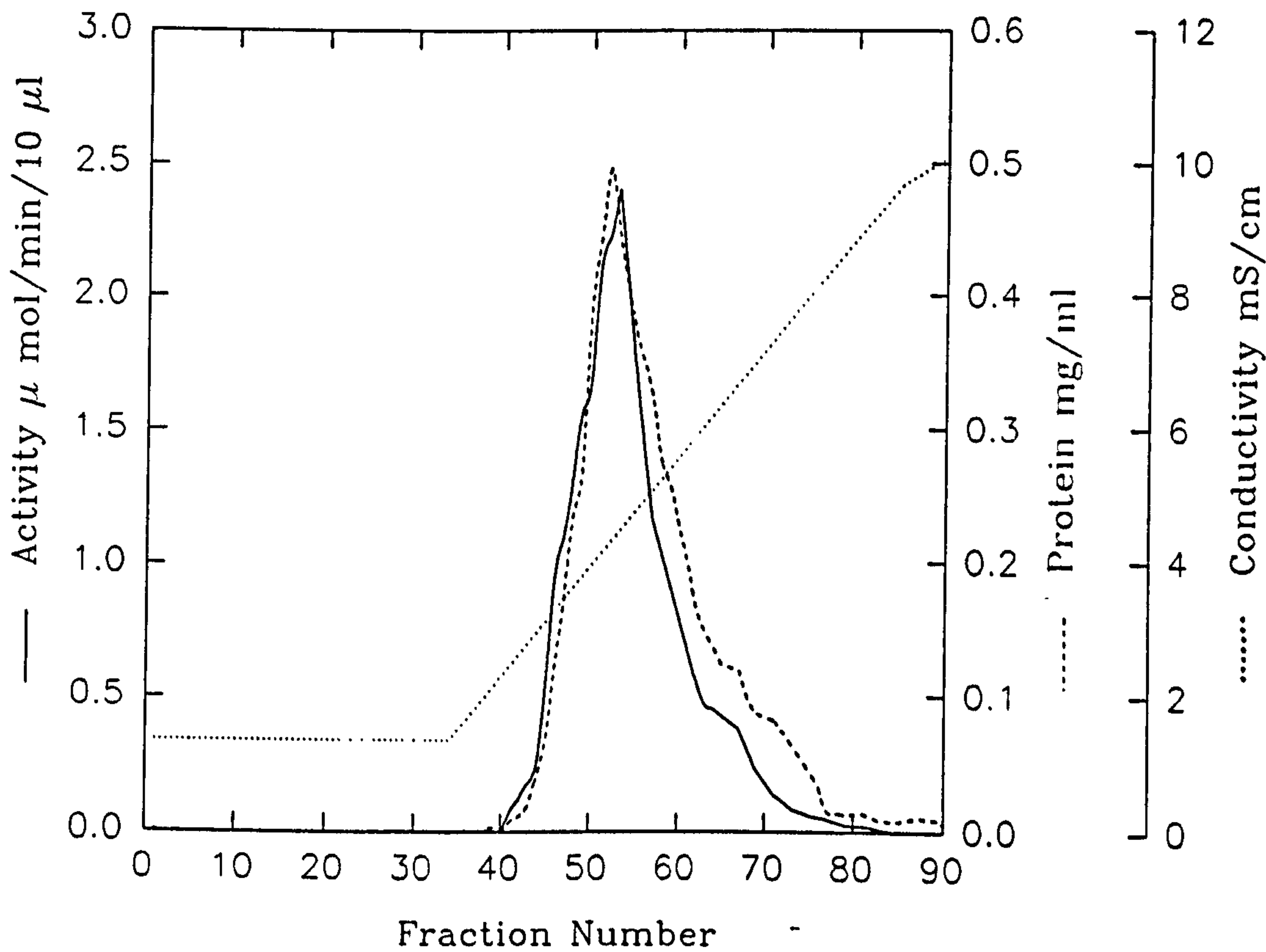
3.3.1. PURIFICATION OF *Est* α 2 AND *Est* β 2

Using the purification procedures described in sections 3.2.1. and 2.2.8., esterases *Est* α 2 and *Est* β 2 could be separated from all contaminating proteins. Chromatography profiles have already been presented in section 2.3.3. for the Q-Sepharose step (FIGURE 2.7) in which *Est* α 2, *Est* β 1 and *Est* β 2 eluted together, the phenyl-Sepharose step (FIGURE 2.8) in which *Est* α 2 eluted in a separate peak to the esterases β which eluted together, and for the hydroxyapatite step (FIGURE 2.9) in which the esterases β again eluted together. The chromatography profile for the hydroxyapatite step of the *Est* α 2 purification is shown in FIGURE 3.1. Data for representative procedures for the purification of *Est* α 2 and *Est* β 2 have already been presented with those for *Est* β 1 in TABLE 2.2. Multiple preparations routinely gave approximately 30% final recovery (as a percentage of the total esterase activity of

FIGURE 3.1

Elution profile of hydroxyapatite chromatography for esterase Est α 2.

1 ml fractions of the elution were collected. The sample containing the esterase was applied to the column in 10 mM phosphate buffer (pH 6.8) and eluted with a 10-200 mM phosphate buffer gradient.

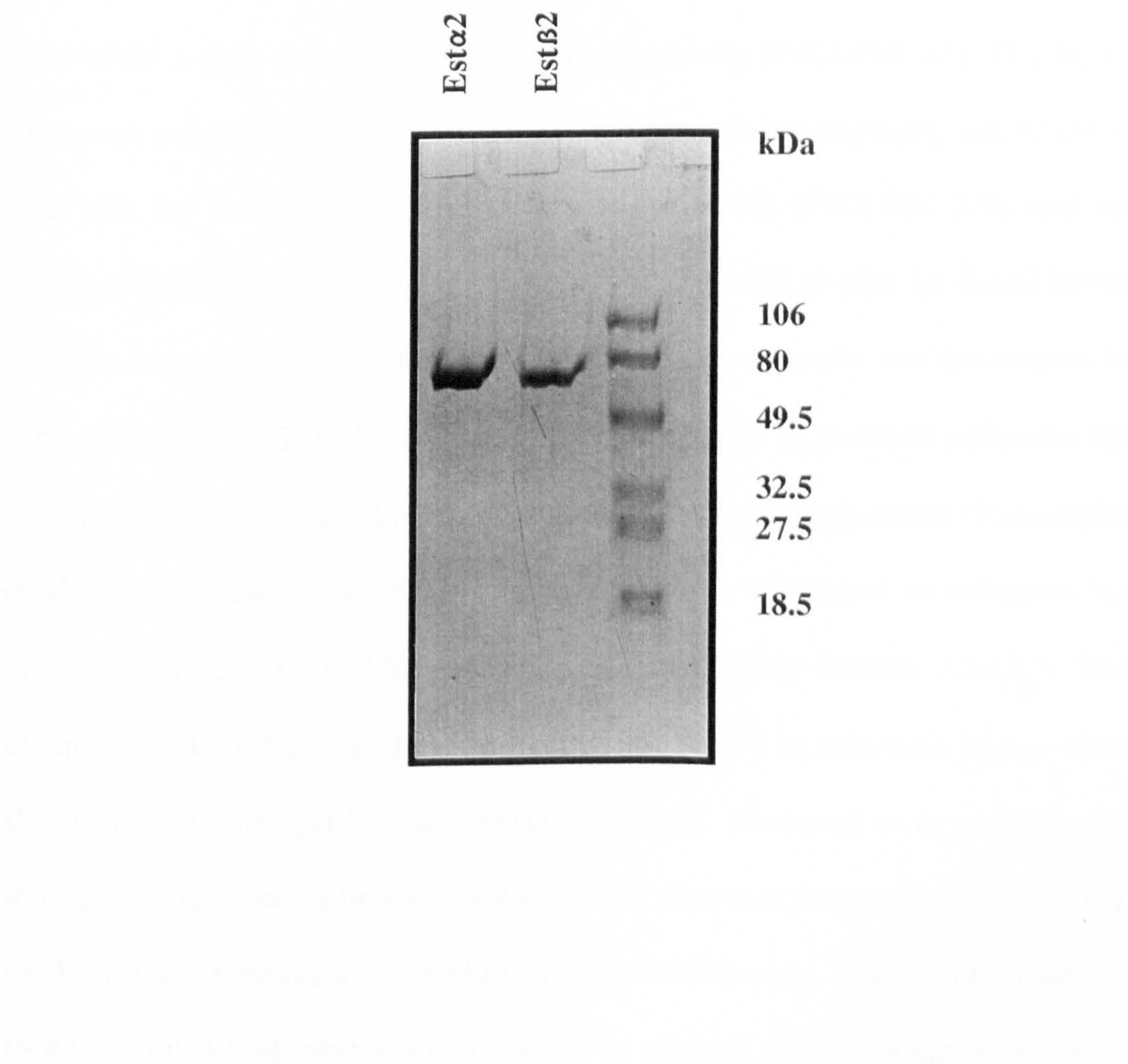


the crude homogenate) and 540-fold purification of Est α 2 and 1.4% final recovery and 55-70-fold purification of Est β 2. Using the outlined procedures approximately 400-500 μ g of purified Est α 2 and 150-250 μ g of Est β 2 were obtained from 15 g wet weight of larvae. Purity was checked after the completion of each procedure by running aliquots of the respective enzyme on SDS-PAGE (FIGURE 3.2).

As mentioned in section 3.1., *est α 2* and *est β 2* are always found co-amplified (Raymond *et al.*, 1991), and there is data to show that these two esterase genes sit on the same amplicon (Vaughan, 1995). However, there is little data on the expression of the two genes in the mosquito. Following separation of Habana Est α 2 from Est β 1 and Est β 2 by phenyl-Sepharose chromatography, the recovered enzymatic protein ratio was about 68% Est α 2 and 32% Est β 1/Est β 2. Assuming the relative proportions of Est β 1 and Est β 2 recovered following phenyl-Sepharose are the same as those recovered from the Prep-Cell, the 32% of recovered Est β can be divided into 6% Est β 1 and 26% Est β 2. The proportions of Est α 2 and Est β 2 have then to be normalised for the difference in V_{\max} for substrate *p*-nitrophenyl acetate (8.75 x 26 Est β 2: 68 Est α 2), giving a final ratio of 3:1 for the proportion of Est β 2 to Est α 2. This ratio is somewhat higher than was found for the co-elevated PelRR Est α 2¹ and Est β 2¹, the proportions here being calculated to be about 1.5:1 (Karunaratne, 1994). In fact, the ratio of Habana Est β 2 to Est α 2 is likely to be higher than 3:1 in the mosquito, Est β 2 being more labile than Est α 2. Karunaratne (1994) found that, to obtain Est α 2¹ and Est β 2¹ bands of an intensity equal to those in crude homogenates of PelRR

FIGURE 3.2

SDS-PAGE of purified esterases Est α 2 and Est β 2. Molecular weight markers on 4-20% gel are; Phosphorylase B (106 kDa), bovine serum albumin (80 kDa), ovalbumin (49.5 kDa), carbonic anhydrase (32.5 kDa), soybean trypsin inhibitor (27.5 kDa) and lysozyme (18.5 kDa).



larvae, about 3-4 times more purified Est β 2 than Est α 2 had to be applied. If the same increase in the proportion of Est β 2 to Est α 2 is applied to the Habana strain, the ratio in the mosquito could be as high as 8:1.

3.3.2. CHARACTERIZATION OF ESTERASES Est α 2 AND Est β 2

3.3.2.1. Physical Characterization

The monomeric M_r of the purified Est α 2 and Est β 2, estimated from SDS-PAGE were 69.49 ± 0.63 and 69.86 ± 0.12 kDa respectively (FIGURE 3.3). The M_r of the native enzyme as estimated from gel filtration chromatography was 63.09 ± 3.66 kDa for Est α 2 and 63.16 ± 3.76 kDa for Est β 2 (FIGURE 3.4), and on gradient 8-25% PAGE to be 73.52 kDa for Est α 2 and 67.14 kDa for Est β 2 (from a single determination) (FIGURE 3.5). Thus, both esterases are monomers in their active forms. The size estimates are similar both to previous estimates for the same enzymes purified from other strains of *C. quinquefasciatus* (Kettermann *et al.*, 1992; Jayawardena, 1992; Karunaratne *et al.*, 1993) and to estimates for Est β 1 (Fournier *et al.*, 1987; Small *et al.*, 1995) (see section 2.3.4.1.). The estimate for Est α 2 obtained by gradient native PAGE is somewhat higher than that obtained from gel filtration chromatography. Problems were experienced during the experiments to obtain estimates for esterases Est α 2, Est β 1 and Est β 2 by the former method as, on ending electrophoresis runs, it was found that the three esterases had migrated more slowly in relation to the standard proteins

FIGURE 3.3

Subunit molecular weight estimation of purified esterases Est α 2 and Est β 2. R_f values of the SDS-PAGE standard proteins were plotted against their molecular weights (on a log scale). Molecular weights of Est α 2 and Est β 2 were determined by linear regression for each gel.

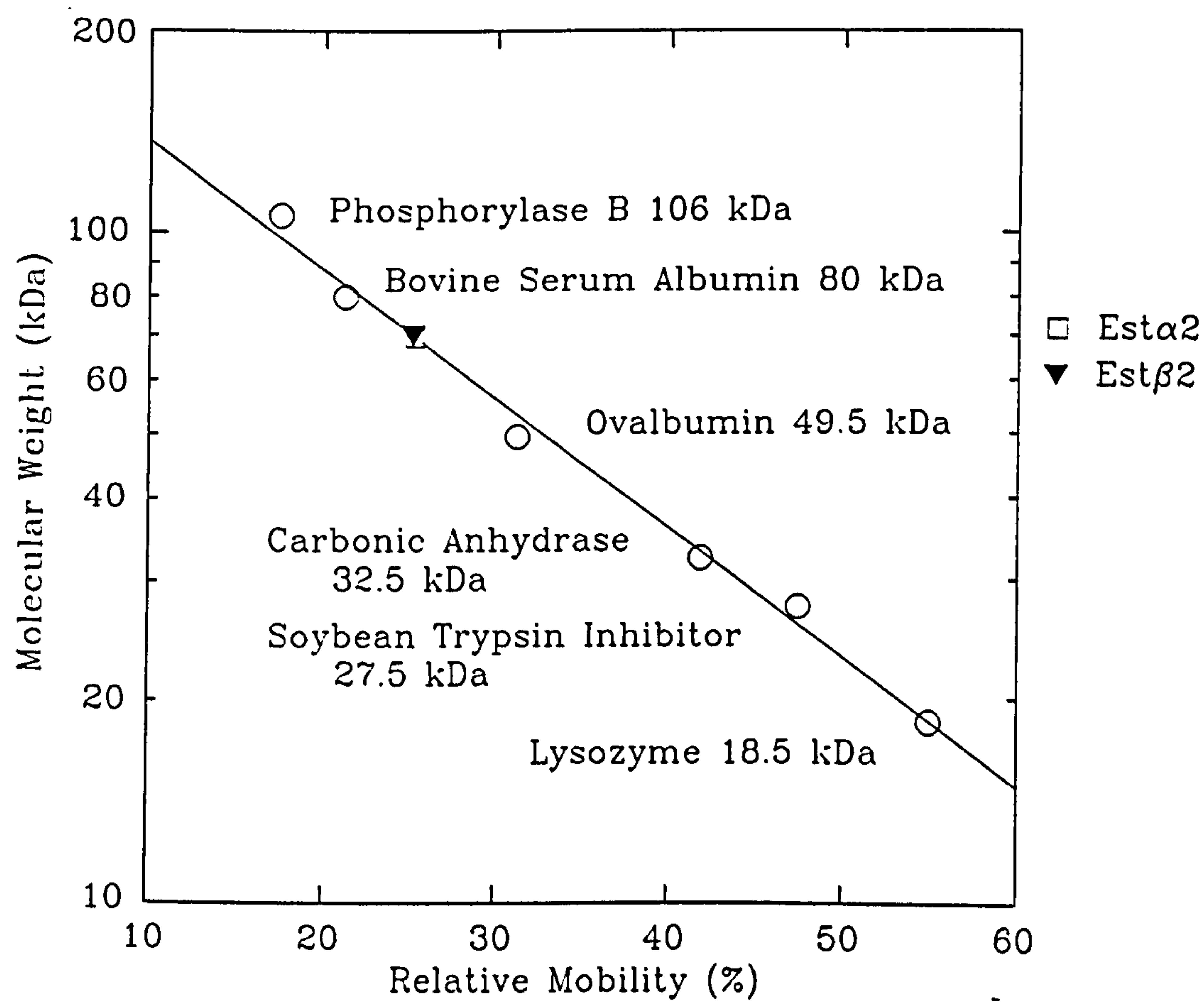


FIGURE 3.4

Native M_r estimation of Est α 2 and Est β 2 by FPLC gel filtration chromatography on Superdex 200. The volume of elution (V_e) divided by the void volume (V_o) for standard proteins was plotted against the molecular weights (on a log scale). Molecular weights of esterases Est α 2 and Est β 2 were determined by linear regression for each column run.

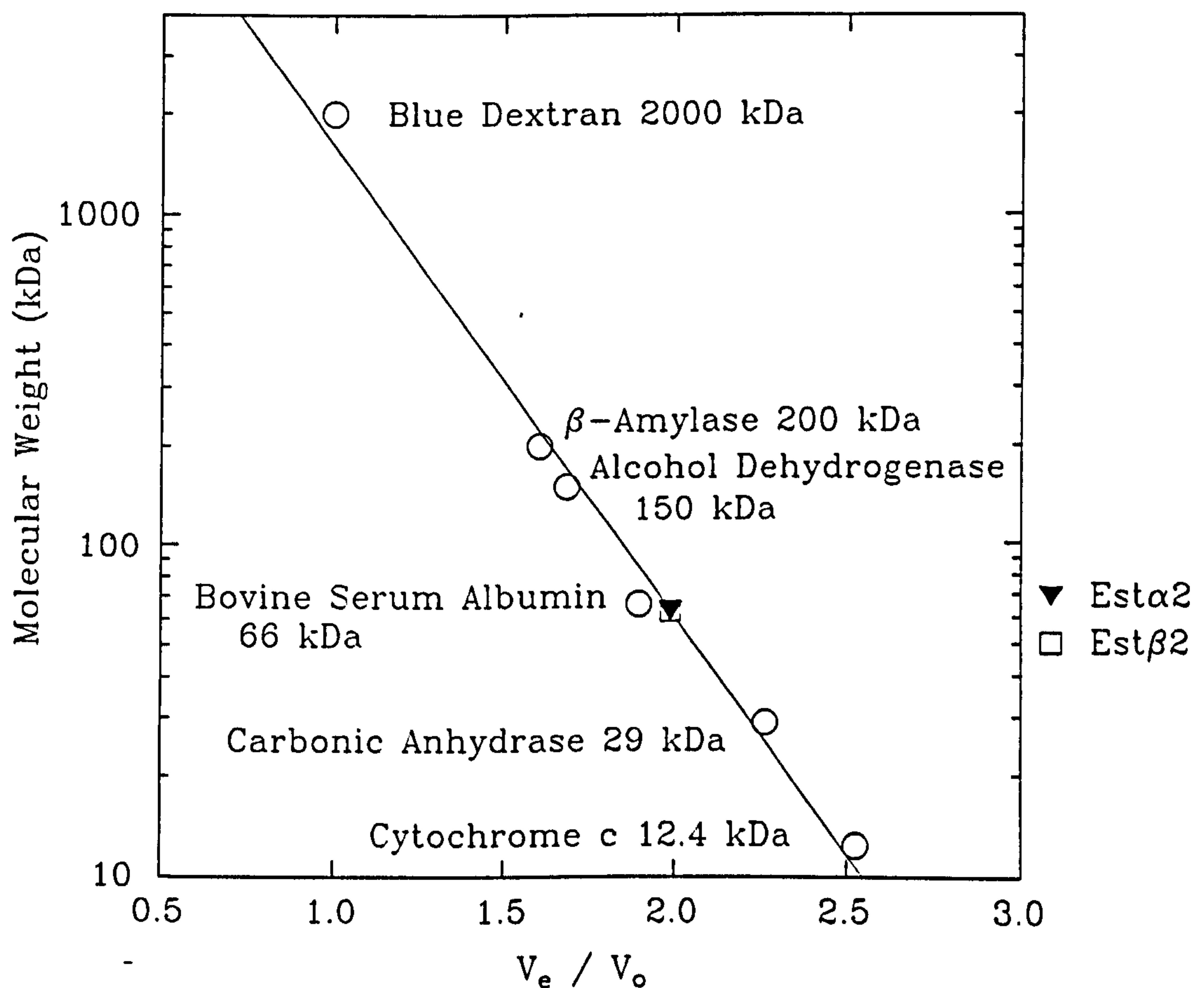
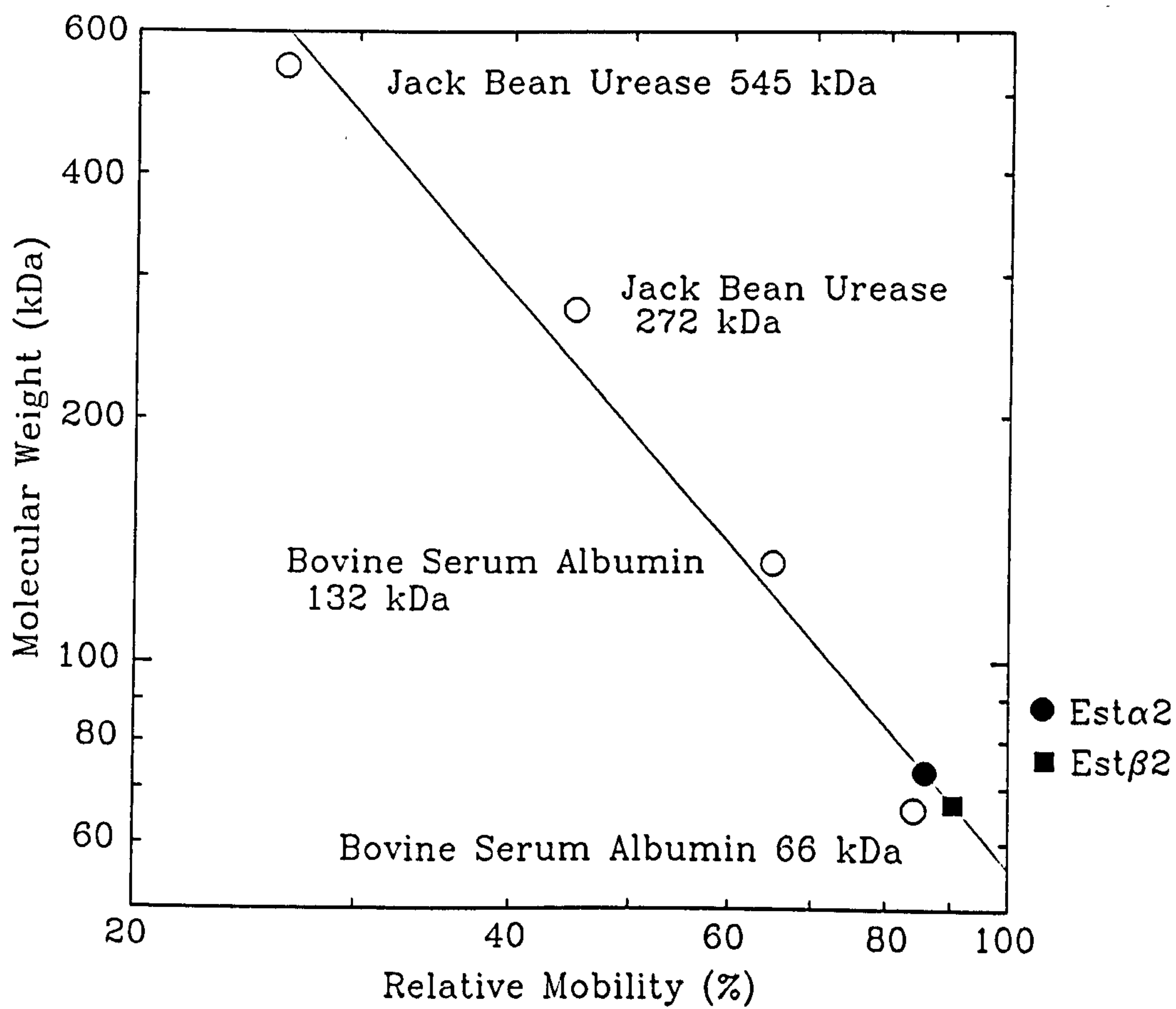


FIGURE 3.5

Native M_r estimation of Est α 2 and Est β 2 by native PAGE on 8-25% gradient gels. R_f values of the native PAGE standard proteins were plotted against their molecular weights (both on log scales). Molecular weights of esterases Est α 2 and Est β 2 were determined by linear regression.

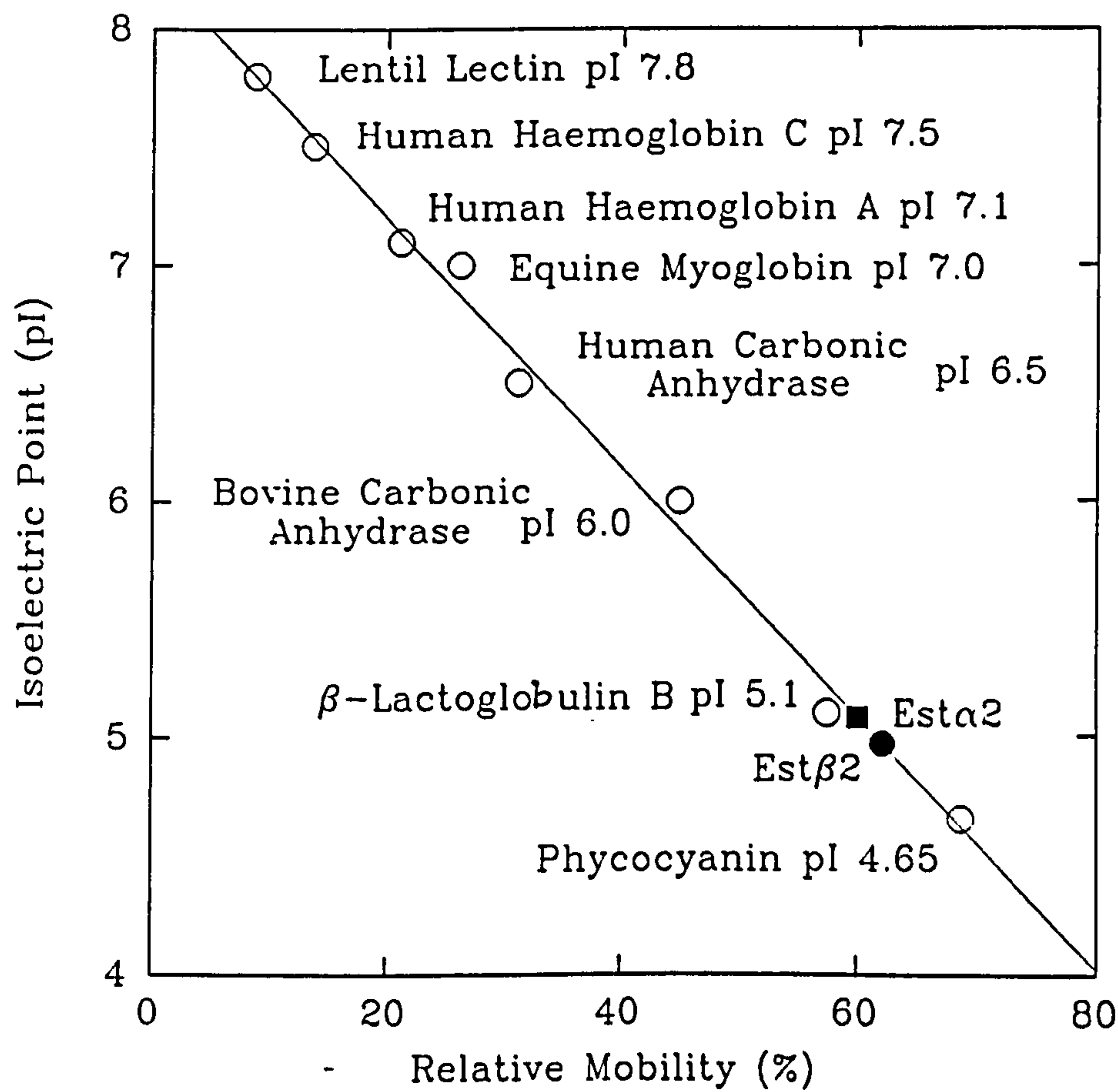


than would be expected from size estimates obtained by gel filtration chromatography. This led to estimates that were higher than expected for protein monomers, but lower than that expected for homodimers (the possibility of their being heterodimers can be discounted based on evidence from SDS/PAGE). In addition, the esterases did not migrate to reach the same relative mobility, as would be expected from the size estimates obtained by gel filtration chromatography but instead remained in the same relative positions as seen on native PAGE gels of a single concentration. This was true even when the electrophoresis run time was increased to its maximum of 160 Vh on the PhastSystem, although after this time the difference in relative mobilities had markedly decreased. For accurate estimates of M_r by gradient native PAGE the protein(s) of interest should be globular or near globular in shape. The fact that the esterases migrated more slowly than their size (estimated by other methods) suggests that these esterases are not globular and are hindered by their shape from migrating through the pores of the gel.

The pIs of Est α 2 and Est β 2, determined by isoelectric focusing on PhastGel IEF, were 5.08 ± 0.01 and 4.83 ± 0.11 respectively (FIGURE 3.6). These are similar to estimates for PelRR Est α 2¹ (pI 5.2) (Kettermann *et al.*, 1992) and Est β 2¹ (pI 5.0) (Karunaratne *et al.*, 1993), and, as with Est β 1 (Small *et al.*, 1995)(see section 2.3.4.1.) are in the range typical of carboxylesterases (Heymann, 1980).

FIGURE 3.6

Estimation of the pIs of esterases Est α 2 and Est β 2 by isoelectric focusing on a gel with a gradient of pH 3-9. The distance of IEF standard proteins from the cathodic end of the gel were plotted against their pIs. The pIs of Est α 2 and Est β 2 were determined by linear regression for each IEF 3-9 and 4-6.5 gel.



3.3.2.2. Chemical characterization

3.3.2.2.1. Influence of Effectors

The influence of several metal ions, including a series of transition metal ions, together with some known carboxylesterase effectors on the activity of purified Habana esterases Est α 2 and Est β 2 can be seen in TABLE 3.1. Like the PelRR esterases Est α 2¹ and Est β 2¹ (Ketterman *et al.*, 1992; Karunaratne *et al.*, 1993) both of these esterases were inhibited by Hg²⁺, although the inhibition of the Habana Est β 2 was greater than was found for the PelRR Est β 2¹. It is known from their amino acid sequences that esterases of type Est α 2 and Est β 2 are both 'B'-type serine hydrolases [according to Aldridge's classification (1953a; 1953b)] (Vaughan *et al.*, 1995; Vaughan & Hemingway, 1995). The Hg²⁺ inhibition is therefore probably through interaction with a thiol group involved either with catalysis or in the conformational stability of the enzymes. As with the Habana Est β 1 (section 2.3.4.2.1.; Small *et al.*, 1995), Est β 2 was inhibited by 1 mM concentrations of all metal ions tested. This is in contrast to the PelRR Est β 2¹ (Karunaratne *et al.*, 1993), which was unaffected by Mg²⁺, Ca²⁺ and Zn²⁺, and was only partially inhibited by Mn²⁺. Both esterases were inhibited by Cu²⁺, Habana Est β 2 to a lesser extent than PelRR Est β 2¹. For the effect of metal ions on other esterases see section 2.3.4.1.1. In using a range of transition metal ions in this effector study it was intended to investigate variability in their effects on esterase activity as a tool for characterisation. As can be seen above, the effects of these metal ions on the Est α 2s and Est β 2s was indeed found to vary. Whilst

TABLE 3.1. Influence of effectors on the activity of purified Habana esterases Est α 2 and Est β 2

Effectors were individually pre-incubated with the purified enzyme for 30 min at 22 °C. Esterase activity was then measured with 1 mM *p*-nitrophenyl acetate in the presence of the relevant effector. Activities for the purified PeIRR esterases Est α 2¹ and Est β 2¹ are presented for comparison (Ketterman *et al.*, 1992; Karunaratne *et al.*, 1993). The data are means \pm standard deviations.

Effector	Concentration	% Control Activity			
		Habana Est α 2	PeIRR Est α 2 ¹	Habana Est β 2	PeIRR Est β 2 ¹
MgCl ₂	1.0 mM	104.14 \pm 5.53	---	23.96 \pm 1.81	113.1
CaCl ₂	1.0 mM	102.55 \pm 5.41	---	26.40 \pm 1.15	105.9
MnCl ₂	1.0 mM	98.00 \pm 3.44	---	33.29 \pm 3.52	96.7
FeCl ₂	1.0 mM	101.42 \pm 3.99	---	19.00 \pm 1.36	---
CoCl ₂	1.0 mM	99.86 \pm 4.75	---	21.34 \pm 1.67	---
NiCl ₂	1.0 mM	100.59 \pm 2.62	---	21.03 \pm 1.20	---
CuCl ₂	1.0 mM	37.40 \pm 0.25	---	25.60 \pm 3.13	4.8
ZnCl ₂	1.0 mM	62.76 \pm 1.72	---	5.27 \pm 0.51	102.3
CdCl ₂	1.0 mM	3.91 \pm 0.01	---	0.44 \pm 0.06	---
HgCl ₂	1.0 mM	0	0.5	0.21 \pm 0.09	1.7
EDTA	1.0 mM	99.12 \pm 6.02	3.3	28.93 \pm 3.05	104.1
Bis-(<i>p</i> -nitrophenyl) phosphate	0.1 mM	0.16 \pm 0.06	0.6	0.56 \pm 0.09	5.9
Paraoxon	0.1 μ M	0.29 \pm 0.01	---	11.53 \pm 0.61	51.3
	0.1 mM	0.18 \pm 0.01	0.6	0.57 \pm 0.08	5.9

the most important characteristic of these enzymes must be their interaction with insecticides, it is possible that this variation in the effects of transition metal ions maybe exploited where other characteristics of enzymes do not enable them otherwise to be distinguished. Both Habana Est α 2 and Est β 2, in common with Habana Est β 1 (section 2.3.4.2.1.; Small *et al.*, 1995) and the PelRR amplified esterases (Kettermann *et al.*, 1992; Karunaratne *et al.*, 1993), were inhibited by paraoxon placing them with the 'B'-type esterases under the classification of Aldridge (1953a; 1953b; 1993). The PelRR Est β 2¹ was less sensitive to inhibition by paraoxon than PelRR Est α 2¹ and the Habana esterases at both concentrations used. All these esterases were inhibited by bis(*p*-nitrophenyl) phosphate which has been shown to be a specific inhibitor of rat carboxylesterases (Brandt *et al.*, 1980), in both *in vivo* and *in vitro* studies. The chelating agent EDTA had no significant effect on Habana Est α 2 activity but decreased Habana Est β 2 activity to 29% of the control. This result is the opposite of that reported for the PelRR Est α 2¹ (Kettermann *et al.*, 1992) and Est β 2¹ (Karunaratne *et al.*, 1993), the former being inhibited and the latter being completely uninhibited by EDTA. Inhibition by EDTA implies the involvement of a metal ion in catalysis. Therefore, these results suggest a fundamental difference between catalysis in the Habana esterases and their PelRR equivalents. Unfortunately, the PelRR esterases were unavailable for comparison at the time the effector studies were carried out on the Habana esterases. The chemicals and procedures used in the studies of all four esterases were identical apart from the buffers used as a vehicle for the EDTA which in the case of the PelRR esterase study was 50 mM phosphate buffer (pH 7.4) and in the case of the Habana esterase study was 25 mM Bistris propane (pH 7.4). It is

unlikely, however, that the difference in buffers would have influenced the result. It is clear that the effect of EDTA and the involvement of metal ions in catalysis needs to be examined further.

3.3.2.2.2. *Substrate specificity*

The Michaelis constants (K_m s) and V_{max} s, were determined for the hydrolysis of the substrates *p*-NPA and *p*-nitrophenyl caproate by the Habana esterases Est α 2 and Est β 2 (TABLE 3.2). Data for PelRR Est α 2¹ and Est β 2¹ (Karunaratne *et al.*, 1993) are presented for comparison. The V_{max} s for the Habana esterases were higher for both substrates than their PelRR equivalents but their K_m s were not significantly different. The values for V_{max} for the Habana esterases fall within the range found for their equivalents purified from other strains of *C. quinquefasciatus* from Tanzania and Saudi Arabia (Karunaratne, 1994). The values for both constants were similar for the Habana Est β 2 and Est β 1 (section 2.3.4.2.2.; Small *et al.*, 1995). As with the PelRR esterases, and the Habana Est β 1 (section 2.3.4.2.2.; Small *et al.*, 1995), Habana Est α 2 and Est β 2 showed a higher affinity towards the *p*-nitrophenyl caproate (C_6) than the *p*-NPA (C_2). The rates of hydrolysis of both substrates is higher for the esterases Est α 2 than for the esterases Est β 2. The K_m s and V_{max} s for the Habana Est α 2 and Est β 2 hydrolysis of *p*-NPA are similar to those reported for some other insect and vertebrate esterases (see section 2.3.4.2.2).

TABLE 3.2. Substrate interactions of purified Habana carboxylesterases Estα2 and Estβ2

The rate of hydrolysis of both substrates used was measured at 405 nm. A unit corresponds to the hydrolysis of 1 μmol of substrate in 1 min under the assay conditions stated. The Michaelis constants and V_{\max} s for the purified PelRR esterases Estα2¹ and Estβ2¹ are presented for comparison (Karunaratne *et al.*, 1993). The data are means ± standard deviations.

Esterase	Substrate...	<i>p</i> -nitrophenyl acetate		<i>p</i> -nitrophenyl caproate	
	Parameter...	V_{\max} (units/mg)	K_m (μ M)	V_{\max} (units/mg)	K_m (μ M)
Habana Est α 2		706.5 \pm 8.5	219.6 \pm 24.2	1593.6 \pm 17.2	31.5 \pm 2.5
PelRR Est α 2 ¹		472.0 \pm 51.4	145.8 \pm 45.0	788.2 \pm 74.8	35.4 \pm 9.7
Habana Est β 2		80.7 \pm 1.8	100.3 \pm 12.4	96.7 \pm 4.4	10.3 \pm 4.4
PelRR Est β 2 ¹		63.4 \pm 0.8	140.0 \pm 50.0	83.3 \pm 9.8	17.0 \pm 6.0

V_{\max} and K_m values for Habana Estα2 and Estβ2 were calculated from three replicates, each replicate using enzymes from a different purification

3.3.2.2.3. Interaction of purified Habana esterases Est α 2 and Est β 2 with insecticides

The bimolecular rate constants, k_a s, for the interaction of various insecticides with purified Habana esterases Est α 2 and Est β 2 are presented in TABLE 3.3. The k_a s for the purified PelRR esterases Est α 2¹ and Est β 2¹ (Karunaratne *et al.*, 1993) are also presented for comparison. The only significant differences between the k_a s of the Habana and PelRR Est α 2s were for their interactions with chlorpyrifos-oxon and propoxur, the k_a of the Habana Est α 2 being higher in both cases. The k_a s of Habana Est β 2 with malaoxon were lower, and with fenitrooxon were higher, than for the PelRR Est β 2¹. Otherwise, there were no significant differences between the k_a s of the two Est β 2s. The interactions of the Habana Est α 2 and Est β 2 were very similar except for their interactions with fenitrooxon, where the k_a of the Est α 2 was approximately 4-fold higher, and with chlorpyrifos-oxon, where its k_a was approximately 2-fold lower than the k_a s for Est β 2. Comparing the two Est α 2s with the Est β 2s, there was no significant difference between any of the k_a s with paraoxon and, in general, no major differences were observed for interaction with any of the other insecticides used indicating that the two esterase types probably play a similar role in organophosphate resistance. The order of increasing reactivity for both the Habana Est α 2 and Est β 2, as with other Est α 2s and Est β 2s purified from other organophosphate resistant strains and also the non-amplified Est α 3 and Est β 1³ purified from the susceptible PelSS strain, was propoxur, malaoxon, fenitrooxon, paraoxon, chlorpyrifos-oxon (Karunaratne *et al.*, 1993; Ketterman *et al.*, 1993; Jayawardena *et al.*, 1994; Hemingway *et al.*, 1993; Karunaratne *et al.*, 1995a). In the other resistant strains the order of the k_a s was generally reflected in the insecticide

TABLE 3.3. The kinetic constant k_a for insecticide interactions with purified Habana esterases Est α 2 and Est β 2

Enzyme activity was measured by the assay of 1 mM *p*-nitrophenyl acetate hydrolysis at 22 °C. The k_a values for the purified PeIRR esterases Est α 2¹ and Est β 2¹ are presented for comparison (Karunaratne *et al.*, 1993). The data are means \pm standard deviations. In the same row, different superscript letters indicate a significant difference ($P<0.05$).

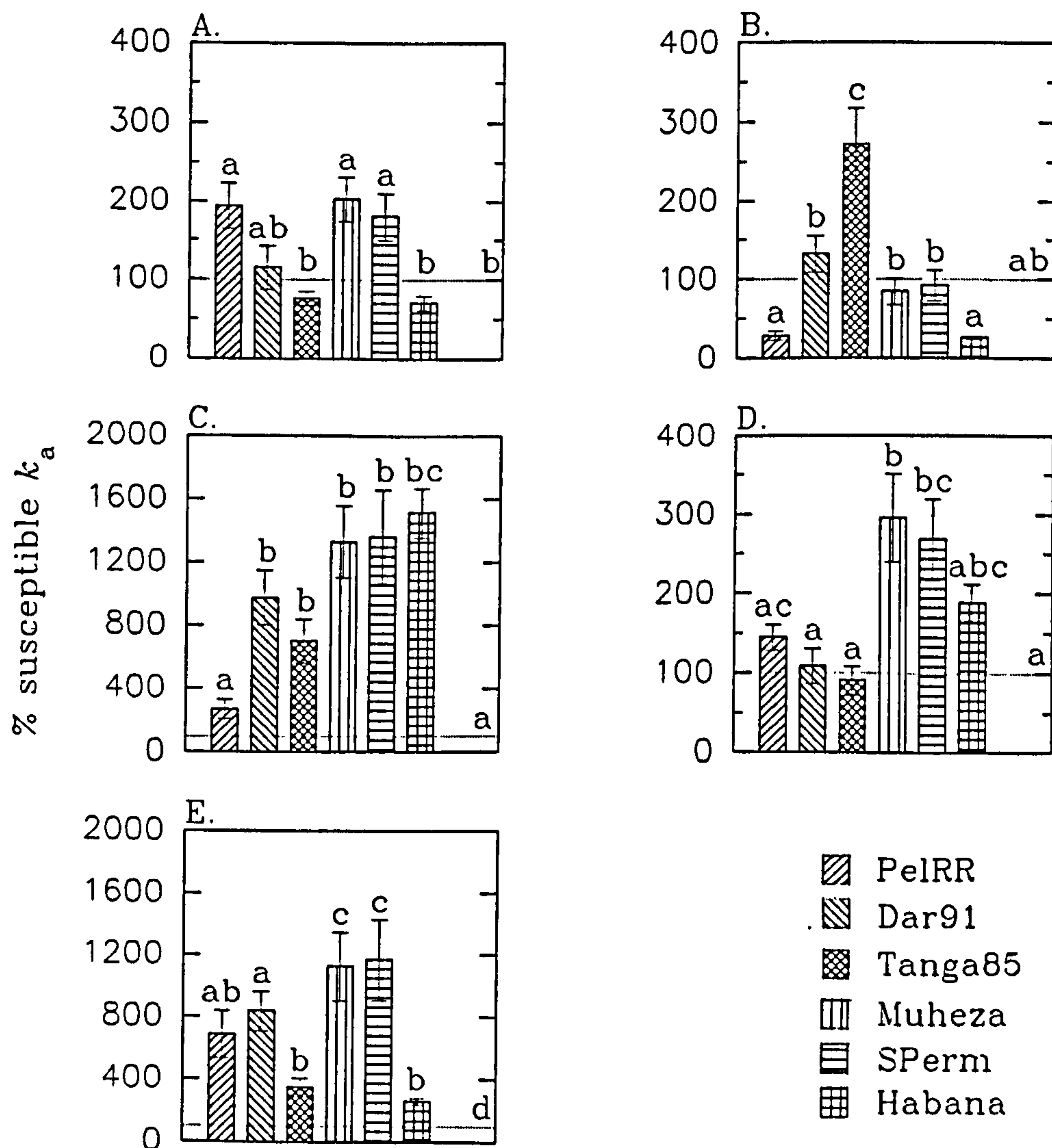
Insecticide	$10^{-5} \times k_a \text{ (M}^{-1} \text{ min}^{-1}\text{)}$				
	Habana Est α 2	PelRR Est α 2 ¹	Habana Est β 2	PelRR Est β 2 ¹	
Malaoxon [*]	0.08 \pm 0.01 ^a	0.17 \pm 0.07 ^{ab}	0.14 \pm 0.02 ^a	0.50 \pm 0.17 ^b	n=31
Fenitrooxon [*]	0.89 \pm 0.05 ^a	0.91 \pm 0.37 ^{ab}	0.20 \pm 0.04 ^b	1.73 \pm 0.60 ^c	n=33
Chlorpyrifos-oxon [*]	807 \pm 82.8 ^a	145.3 \pm 59.7 ^b	1446 \pm 38.2 ^c	1550 \pm 140 ^c	n=12
Paraoxon [*]	247 \pm 30.7 ^a	178 \pm 47.7 ^a	192 \pm 45.0 ^a	170 \pm 53 ^a	n=28
Propoxur [†]	0.0047 \pm 0.0004 ^a	0.012 \pm 0.003 ^b	0.0037 \pm 0.0008 ^a	0.0052 \pm 0.0017 ^a	n=26
[*] Organophosphate					
[†] Carbamate					

cross-resistance spectra with the phosphorothionate form of the insecticides and with propoxur, with the highest resistance ratio (ratio of LD50 of the resistant strain to the LD50 of the susceptible PelSS strain) being with chlorpyrifos going down to the lowest with propoxur (Hemingway *et al.*, 1993). The cross-resistance of the Habana strain to different organophosphorus insecticides was not investigated in this study. The resistance ratio of the Habana strain was found to be higher with malathion than with propoxur (see TABLE 2.1). However, it must be remembered that the other elevated esterases and the altered acetylcholinesterase mechanism would all have influenced this result which must therefore be interpreted with caution. A comparison between the k_a s for the Habana Est α 2, Est β 2 and Est β 1 (see TABLE 2.5.) revealed that Est β 1 had a higher k_a with both malaoxon and fenitrooxon than the other two elevated esterases. The only insecticide with which Est β 1 had a k_a significantly lower than Est α 2 and Est β 2 was with propoxur. These data suggest, therefore, that the hypothesis that the co-elevated Est α 2 and Est β 2 have spread into *C. quinquefasciatus* populations in Cuba by virtue of a higher efficacy for the binding of OPs than Est β 1 is incorrect. A comparison between the k_a s for the Habana Est α 2 and those for Est α 2s purified from other organophosphate resistant strains from Tanzania (Muheza and Tanga '85) and Saudi Arabia (SPerm) (Karunaratne *et al.*, 1993; Ketterman *et al.*, 1993; Jayawardena *et al.*, 1994; Karunaratne *et al.*, 1995a) is presented graphically in FIGURE 3.7. Values for PelRR are also included. This figure expresses the k_a s for Est α 2 purified from each of the resistant strains, and with each of the insecticides, as a percentage of the value of the non-amplified Est α 3 purified from the susceptible PelSS strain (Hemingway *et al.*, 1993; Karunaratne *et al.*, 1995a). For malaoxon and fenitrooxon

The statistical test used to determine the significance of differences between mean values was that described in section 2.3.5.2.3. This test was carried out on the original, untransformed data

FIGURE 3.7

Bimolecular rate constants (k_a) for (A.) malaoxon, (B.) fenitrooxon, (C.) chlorpyrifos-oxon, (D.) paraoxon, and (E.) propoxur with Habana Est α 2 and a range of Est α 2s from resistant strains of *C. quinquefasciatus* (Karunaratne *et al.*, 1993; Ketterman *et al.*, 1993; Jayawardena *et al.*, 1994; Karunaratne *et al.*, 1995a) expressed as a percentage of the value of non-elevated Est α 3 from the insecticide suseceptible PelSS strain (Hemingway *et al.*, 1993; Karunaratne *et al.*, 1995a). Different letters indicate a significant difference ($P < 0.05$).



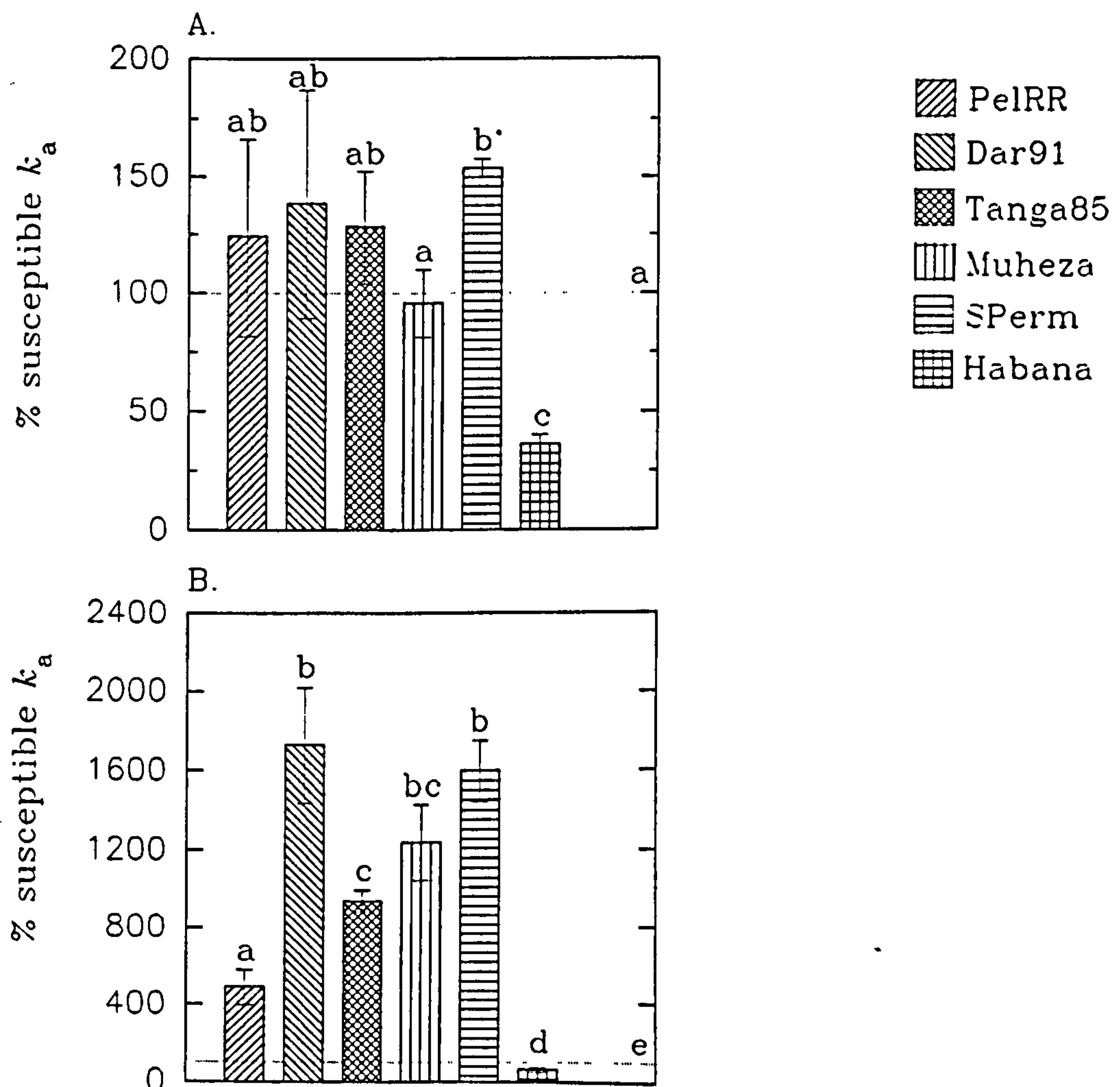
the Habana Est α 2 was not significantly different from the susceptible Est α 3, and for these insecticides, together with propoxur, was the lowest of the resistant strains though not significantly so. The largest difference between values of k_a between Habana and the other resistant strains was with fenitrooxon, there being a 10.5 fold difference between the Habana k_a and that for Tanga85. Taken together, the graphs show that the Habana Est α 2 can readily be distinguished from Est α 2 purified from other resistant strains on the basis of its interaction with the different insecticides. The k_a s for the Habana Est β 2 and Est β 2s purified from the same organophosphate resistant strains mentioned above are expressed in the same way with the insecticides malaoxon and fenitrooxon in FIGURE 3.8. Here k_a s for the Est β 2s from the resistant strains were expressed as a percentage of those for the non-amplified Est β 1³ purified from the PelSS strain (Hemingway *et al.*, 1993; Karunaratne *et al.*, 1995a). With both insecticides the Habana Est β 2 is significantly lower than all other Est β 2s and the susceptible Est β 1³. The k_a s for chlorpyrifos-oxon, paraoxon and propoxur were significantly higher than the susceptible Est β 1³, but not significantly different to the other Est β 2s (data not presented). As with the Est α 2s, the largest difference between values of k_a for Habana and the other resistant strains was with fenitrooxon, there being a 28 fold difference between the Habana k_a and that for Dar91.

As an explanation of the variation in the k_a s of the enzyme preparations from PelRR and other strains having co-elevated Est α 2 and Est β 2, it was suggested that this variation might be due to minor contamination with different non-elevated esterases α or β , and/or to different levels of amplification of the *est* α 2 and *est* β 2

The statistical test used to determine the significance of differences between mean values was that described in section 2.3.5.2.3. This test was carried out on the original, untransformed data

FIGURE 3.8

Bimolecular rate constants (k_a) for (A.) malaoxon and (B.) fenitrooxon with Habana Est β 2 and a range of Est β 2s from resistant strains of *C. quinquefasciatus* (Karunaratne *et al.*, 1993; Ketterman *et al.*, 1993; Jayawardena *et al.*, 1994; Karunaratne *et al.*, 1995a) expressed as a percentage of the value of non-elevated Est β 1³ from the insecticide suseptible PelSS strain (Hemingway *et al.*, 1993; Karunaratne *et al.*, 1995a). Different letters indicate a significant difference ($P < 0.05$).



genes with concomitant variation in the proportion of non-elevated esterase present (Karunaratne *et al.*, 1995a) (see section 3.1). It has been shown by restriction fragment analysis of non-amplified *est* α and *est* β alleles in field populations that they exhibit a high level of polymorphism (Raymond *et al.*, 1991) which might in turn result in variation in the k_a s for the expressed non-amplified proteins. It is likely also that there was variation in the degree of amplification in the different strains of organophosphate resistant strains used in the characterization studies. Whilst levels of amplification have not been examined in strains having co-amplified *est* α 2/*est* β 2, variation in the degree of amplification has been shown for the *est* β 1 gene in *C. quinquefasciatus* field populations (Raymond *et al.*, 1989) and also for the genes coding for elevated esterases E4 and FE4 in field populations of *Myzus persicae* (Field *et al.*, 1988). To date, the hypothesis of Raymond *et al.* (1991) for the spread of co-amplified *est* α 2/*est* β 2 worldwide by migration, together with the hypothesis of Karunaratne *et al.* (1995) to explain the variation in insecticide interactions of Est α 2 and Est β 2 purified from the various organophosphate resistant strains, best explain the available molecular biological and biochemical data. What now needs to be examined, by molecular biological investigation of the degree of amplification of the co-amplified esterase genes and their expression (for possible post-transcriptional and post-translational modifications), and by biochemical investigation of the insecticide interactions of other non-elevated esterases α and β , is whether the hypothesis of Karanaratne *et al.* (1995) can fully explain the sometimes marked variations in k_a s of the purified elevated esterases as exemplified through comparison of the Habana Est α 2 and Est β 2 and their equivalents in this study.

3.4. SUMMARY

The elevated esterases Est α 2 and Est β 2 were successfully purified from fourth instar larvae of the Habana strain of *C. quinquefasciatus* using methodologies adapted from those previously used to purify other Est α 2s and Est β 2s (Kettermann *et al.*, 1992; Jayawardena, 1992; Karunaratne *et al.*, 1993). The physical characteristics of these esterases were similar to each other, to those for the same enzymes purified from the PelRR strain (Kettermann *et al.*, 1992; Jayawardena, 1992; Karunaratne *et al.*, 1993), and to those for Est β 1 (Fournier *et al.*, 1987; Small *et al.*, 1995; section 2.3.4.2.1.). Differences were found between the sensitivity of the Habana Est β 2 and PelRR Est β 2¹ esterases to the presence of metal ions. Variation in the effect of EDTA on the Habana esterases when compared to their PelRR equivalents suggested differences in the involvement of metal ions in catalysis. The Habana Est β 2 behaved similarly to the Habana Est β 1 (Small *et al.*, 1995; section 2.3.4.2.2.) with all effectors tested. Values of V_{\max} for the hydrolysis of the substrates *p*-NPA and *p*-nitrophenyl caproate were higher for the Habana Est α 2 and Est β 2 than for their PelRR equivalents (Karunaratne *et al.*, 1993) but were within the range for those purified from other organophosphate resistant strains (Karunaratne, 1994). Values of the Michaelis constant, K_m , for the Habana esterases were not significantly different to their equivalents purified from any of the strains having elevated Est α 2 and Est β 2 (Karunaratne *et al.*, 1993; Karunaratne, 1994). The values of both constants for the Habana Est β 2 and Est β 1 (Small *et al.*, 1995; section 2.3.4.2.2.) were not significantly different for either of the substrates used. On the basis of their k_a s, both Habana Est α 2 and Est β 2 could be distinguished from their

equivalents purified from other organophosphate resistant strains. With both malaoxon and fenitrooxon, both Habana Est α 2 and Est β 2 had k_s s lower than non-elevated est α 3 and est β 1³ respectively, purified from the susceptible strain PelSS (Hemingway *et al.*, 1993; Karunaratne *et al.*, 1995a), although this was only statistically significant for the Habana Est β 2. The variation in the k_s s of these enzymes is discussed in the context of the hypotheses of Raymond *et al.* (1991) and Karunaratne *et al.* (1995) which together explain the molecular and biochemical data currently available on these esterases.

CHAPTER 4

Chapter 4

IMMUNOLOGICAL CROSS-REACTIVITY OF AN ANTISERUM RAISED AGAINST PELRR Est α 2¹ WITH ESTERASES OF THE HABANA STRAIN

4.1. INTRODUCTION

In experiments investigating the cross-reactivity of antisera raised against *Culex* esterases, Mouches *et al.* (1987) reported that, on western-blot of crude homogenates of *Culex* mosquitoes containing various esterases α and β , an antiserum raised against denatured esterase Est β 1¹ purified from the Californian TEM-R strain of *C. quinquefasciatus* cross-reacted only with other esterases β (Est β 1¹ and Est β 2 in *C. quinquefasciatus*, and esterase B₃ in *C. tarsalis*), and an antiserum raised against denatured esterase Est α 1 purified from the French S54 strain of *C. pipiens* reacted only with other esterases α (Est α 1 in *C. pipiens*, Est α 2 in *C. quinquefasciatus*, and esterase A₃ in *C. tarsalis*). Their study used the 4-chloro-1-naphthol detection method. Similarly, Poirie *et al.* (1992), examining the cross-reactivity of the TEM-R Est β 1¹ antiserum with immunoblots of crude homogenates of the VIM and CYPRUS strains, both containing esterases Est α 4 and Est β 3, found it to cross-react only with Est β 3. When the dye 3,3'-diaminobenzidine tetrahydrochloride was used for detection of cross-reactivity, a polyclonal antiserum raised against the native esterase Est α 2¹ purified from the PelRR strain of *C. quinquefasciatus* was shown to cross-react with Est α 2¹ but not with Est β 2¹ from the same strain (Jayawardena, 1992). This same detection system was used in the

present study to examine the cross-reactivity of the Est α 2¹ antiserum with western-blots of crude homogenates of individual larvae of the Habana strain containing esterases Est α 2, Est α 3, Est β 1 and Est β 2 plus some, as yet, unclassified esterases α and β .

More recently, the highly sensitive ECL (enhanced chemiluminescence) detection system was used to detect cross-reactivity of the PelRR Est α 2¹ antiserum with esterases α and β in strains of *C. quinquefasciatus* in western-blots and, with the purified esterases, in dot-blots (Karunaratne *et al.*, 1993; Karunaratne, 1994; Karunaratne *et al.*, 1995b). In contrast to previous studies, cross-reactivity was detected between this esterase α antiserum and esterases β , in both western blots and dot blots, although this cross-reactivity was lower than with the esterases α . The ECL detection system was used in the present study to examine the cross-reactivity of this antiserum with dot-blots of different protein concentrations of esterases Est α 2, Est β 1 and Est β 2 purified from the Habana strain.

4.2. MATERIALS AND METHODS

4.2.1. CHEMICALS AND EQUIPMENT

Zeta-Probe GT membrane was purchased from Bio-Rad, U.K. and Polyvinylidene difluoride (PVDF) transfer membrane from Millipore (U.S.A.). The Midget Multiblot electrophoretic transfer unit was purchased from LKB Produkter AB

(Sweden). Anti-rabbit IgG, horseradish peroxidase linked with whole antibody (from donkey) and ECL detection reagents were purchased from Amersham P.L.C. (U.K.). All other chemicals were purchased from Sigma, U.K.

The polyclonal antiserum was raised against purified PelRR esterase Est α 2¹ by standard methods in white New Zealand rabbits (Jayawardena, 1992) and stored at -70°C in 20 μ l aliquots until used.

4.2.2. IMMUNOBLOTTING TECHNIQUES

All the immunoblotting techniques were carried out at room temperature (22°C).

4.2.2.1. Western-blotting

In order to test the cross-reactivity of the polyclonal Est α 2¹ antiserum with all elevated esterases present in the Habana strain, and because not all elevated esterases were present in the same individual, it was first necessary to select homogenates of individuals so that all elevated esterases would be represented on the immunoblot. This was achieved by running aliquots of homogenates on native PAGE gels and staining for esterase activity as described in section 2.2.9.1. Fourth instar larvae were homogenised in 200 μ l of 0.02 M phosphate buffer, pH 7.2, and a 20 μ l aliquot run on a 7.5% acrylamide gel. To preserve esterase activity the homogenates were snap frozen in liquid nitrogen immediately the aliquot had been taken. Selected homogenates were thawed on ice, and aliquots loaded onto 7.5%

acrylamide gels and run as before. Two gels were run in tandem with homogenates loaded in the same sequence on each. Homogenates of PelRR larvae containing Est α 2¹ and Est β 2¹ were run on the same gels for comparison. Equal amounts of crude homogenate protein were loaded (10 μ g for each), dilutions being with 0.02 M phosphate buffer. One gel was stained for esterase activity by the method previously described (see section 2.2.9.1.) and the other prepared for blotting. This gel was placed into transfer buffer (0.025 M Tris, 0.2 M glycine, pH 8.3) for 15 min to equilibrate. While this was in progress a Zeta-Probe GT membrane, three gel-sized Whatman No.1 filter papers and the blotter sponges were equilibrated in the same buffer. The 'blotting sandwich' was assembled on a tray in the following order; one of the blotter sponges, one of the filter papers, the Zeta-Probe GT membrane, the gel, the two remaining filter papers, and finally the second blotter sponge. The sandwich was then checked to see that no air bubbles were trapped between any of the layers and secured with two rubber bands. The sandwich was placed into the tank of the Midget Multiblot transfer unit with the gel cathodic in orientation to the membrane. Proteins were transferred at 200 mA for 2 hours. The position of the gel was then marked on the membrane which was placed into washing buffer (0.05% Tween-20 (v/v) in PBS (80 mM Na₂HPO₄, 20 mM NaH₂PO₄ and 100 mM NaCl, pH 7.5) for 1 hour. After blocking non-specific sites with 5% (w/v) non-fat milk in washing buffer for 2 x 30 min the membrane was incubated for 90 min with the Est α 2¹ antiserum (1:5000 dilution in blocking buffer). The membrane was washed for 4 x 20 min in washing buffer and then incubated with the Anti-rabbit IgG peroxidase conjugate [1:500 dilution in washing buffer] for 1 hour. The washing was then repeated as described above. During the last 20 min

wash the substrate solution was prepared (0.03% (w/v) 3,3'-diaminobenzidine tetrahydrochloride in PBS with 15 μ l of 6% hydrogen peroxide added per 50 ml of this solution). The membrane was placed into this substrate solution and the colour allowed to develop.

4.2.2.2. Dot-Blot Assay

Purified Habana esterases Est α 2, Est β 1 and Est β 2 were diluted with distilled water to obtain the required protein concentrations. A 5 μ l dot for each sample was placed directly onto PVDF membrane. Dilutions of purified PelRR Est α 2¹ were also dotted onto the membrane for comparison. The membrane was then placed in blocking solution (see section 4.2.2.1.) for 2 x 30 min and incubated with the Est α 2¹ antiserum (1:2500 dilution in blocking buffer) for a further hour. After washing in 0.05% (v/v) Tween-20 in PBS (see section 4.2.2.1.), 0.05% (v/v) Tween-20 plus 0.5 M NaCl in PBS and in 0.05% (v/v) Tween-20 in PBS for 5 min each, the membrane was incubated for 1 hour with the Anti-rabbit IgG peroxidase conjugate [1/1000 dilution in 0.05% (v/v) Tween-20, 1% (w/v) non-fat milk in PBS]. The washing was repeated as described above. The membrane was then incubated for about 1 min in the ECL detection reagent and the bound anti-body detected by exposing to hyperfilm ECL for 30 seconds to 2 min.

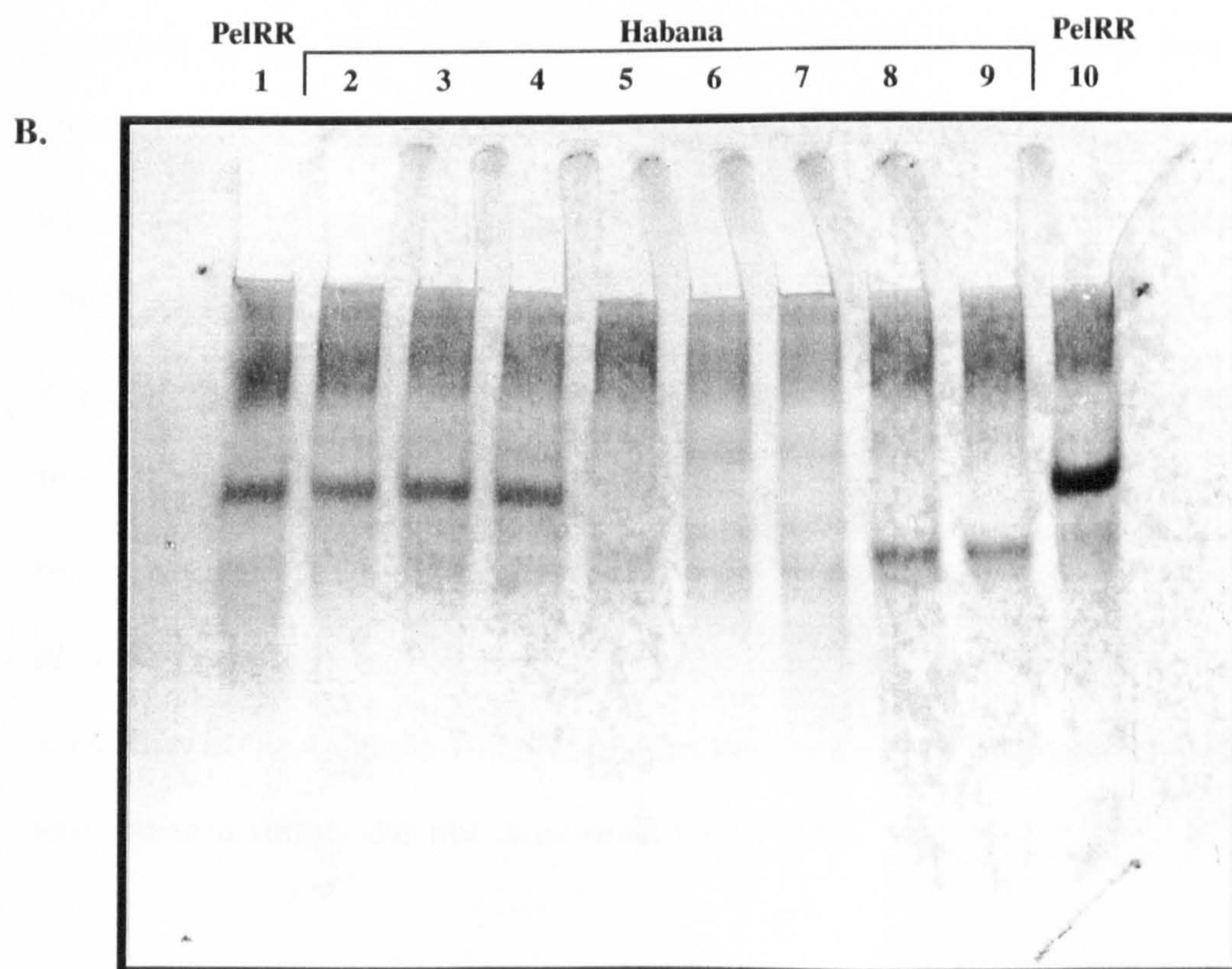
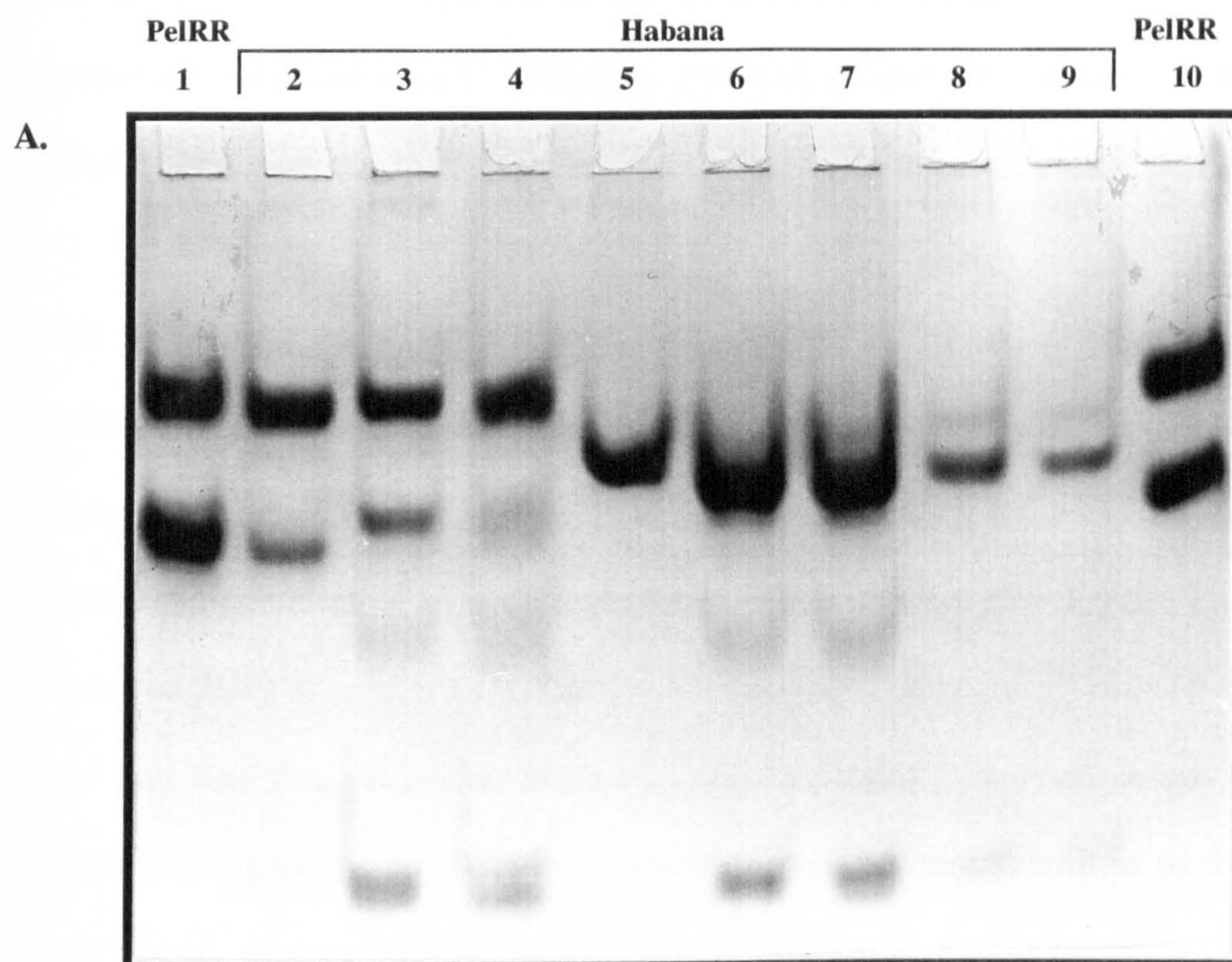
4.3. RESULTS AND DISCUSSION

4.3.1. WESTERN-BLOTTING

To investigate whether the polyclonal antiserum raised against purified Est α 2¹ from the Sri Lankan PelRR strain would cross-react with other esterases α , or with the esterases β , present in the Habana strain, crude homogenates containing esterases Est α 2, Est α 3, Est β 1, Est β 2 and other, as yet unclassified esterases α and β were run on native PAGE gels and proteins electrophoretically transferred under native and denaturing conditions. The 3,3'-diaminobenzidine tetrahydrochloride dye detection method was used for immunodetection, the ECL method being unavailable at the time these experiments were carried out. The esterases β were not observed to cross-react using either method of protein transfer. Esterases α were observed to cross-react in both their native and denatured forms (results not shown). However, a higher level of cross-reactivity could be observed for the native forms, probably because the antiserum had been raised against the native PelRR Est α 2¹. This was also observed by Jayawardena (1992) when examining the cross-reactivity of this antiserum with blots of crude homogenates of PelRR larvae. The results of a western-blot of crude homogenates of Habana larvae containing the esterases mentioned above run on native PAGE and immunodetected with Est α 2¹ antiserum can be seen in FIGURE 4.1 B. A gel run at the same time as the blotted gel, and with the same homogenates, but stained for esterases activity can be seen in FIGURE 4.1 A. Homogenates of PelRR larvae containing Est α 2¹ and Est β 2¹ were included on both the immunoblot and esterase activity gels for comparison. From

FIGURE 4.1

Equal amounts of crude homogenate of Habana individuals (A.) run on a native PAGE gel stained for esterase activity, and (B.) immunodetected on a western-blot with the PelRR Est α 2¹ antiserum. Homogenates of PelRR individuals, possessing elevated esterases Est α 2¹ and Est β 2¹, were included in Lanes 1 and 10 on both the native gel and the immunoblot for comparison. Elevated esterases seen in individuals of the Habana strain on the the native gel and immunoblot, listed in order of R_f, are: Lane 2, Est α 2, Est β 2 and an Est β of high R_f; Lane 3, Est α 2, Est β 2, and an Est α and Est β of high R_f; Lane 4, Est α 2, Est β 2, and an Est α and Est β of high R_f; Lane 5, Est β 1; Lane 6, Est β 1 and an Est α and Est β of high R_f; Lane 7, Est β 1 and an Est α and Est β of high R_f; Lane 8, Est α 3 and an Est β of high R_f; Lane 9, Est α 3 and an Est β of high R_f. The 3,3'-diaminobenzidine tetrahydrochloride dye detection method was used.



the immunoblot it is apparent that both Est α 2 and Est α 3 in the Habana strain cross-react with the Est α 2¹ antiserum and with reactivities similar to the PelRR Est α 2¹ itself showing that they are immunologically closely related.

The highly reactive band of low mobility present at the top of the blot for each individual can be identified as AChE according to the study of Peiris (1989) and Karunaratne *et al.* (1995) who used AChE activity staining to detect this enzyme in crude homogenates of *C. quinquefasciatus* larvae run on native gels. This strong cross-reactivity suggests that AChE too is immunologically closely related to Est α 2¹, and that they contain one or more conserved epitopes. The cross-reactivity of the antiserum can not be due to epitopes containing glycosylated residues, as the Est α 2¹ was found not to interact with Con-A Sepharose suggesting that it is not glycosylated (Karunaratne, 1994). Indeed, analysis of the deduced amino acid sequence of the cDNA of this esterase showed it to have only two possible glycosylation sites, neither of which were conserved in other esterases and both of which were very close to the putative active site serine (Vaughan & Hemingway, 1995). Moreover, the cDNA had no hydrophobic signal sequence suggesting that it is a non-glycosylated protein that is not transported out of the cell, but remains in the cell cytoplasm (Vaughan & Hemingway, 1995). No cross-reactivity was reported between either the Est α 1 or Est β 1 antisera with AChE in the study of Mouches *et al.* (1987) or that of Poirie *et al.* (1992). This may have been due to the low sensitivity of the 4-chloro-1-naphthol detection method used, or possibly because the two antisera simply did not cross-react with AChE.

A faint band with the same relative mobility as the Habana elevated Est α 3 (in lanes 2 and 3, FIGURE 4.1 A.) can be seen on the immunoblot (FIGURE 4.1 B.), this band being present in both the PelRR and Habana strains. A band was found in the same position in blots of homogenates of PelSS individuals (Karunaratne, 1994), this corresponding in position to the non-elevated Est α 3 present in this strain. It is possible that this band in the Habana and PelRR strains corresponds also to a non-elevated Est α 3. The weakly reactive band of high mobility seen on the immunoblot is at the same position as the esterase α seen in the activity-stained native PAGE gel. However, this band can be seen in all homogenates on the immunoblot, including those of PelRR individuals, where no activity can be seen on the native gel. It is unlikely, therefore, that this esterase α of high mobility cross-reacted with the Est α 2¹ antiserum. A faint band can also be seen in individuals of both strains with a mobility intermediate between the AChE and Est α 2, this band not corresponding to any seen on the esterases stained gel. All these weakly reactive bands may correspond to non-elevated esterases α which, because they make up only a very small proportion of the total protein, do not reveal themselves on the esterase activity gel. Alternatively, it maybe that the substrate specificity of these esterases is such that neither α -naphthyl acetate nor β -naphthyl acetate acted as substrates.

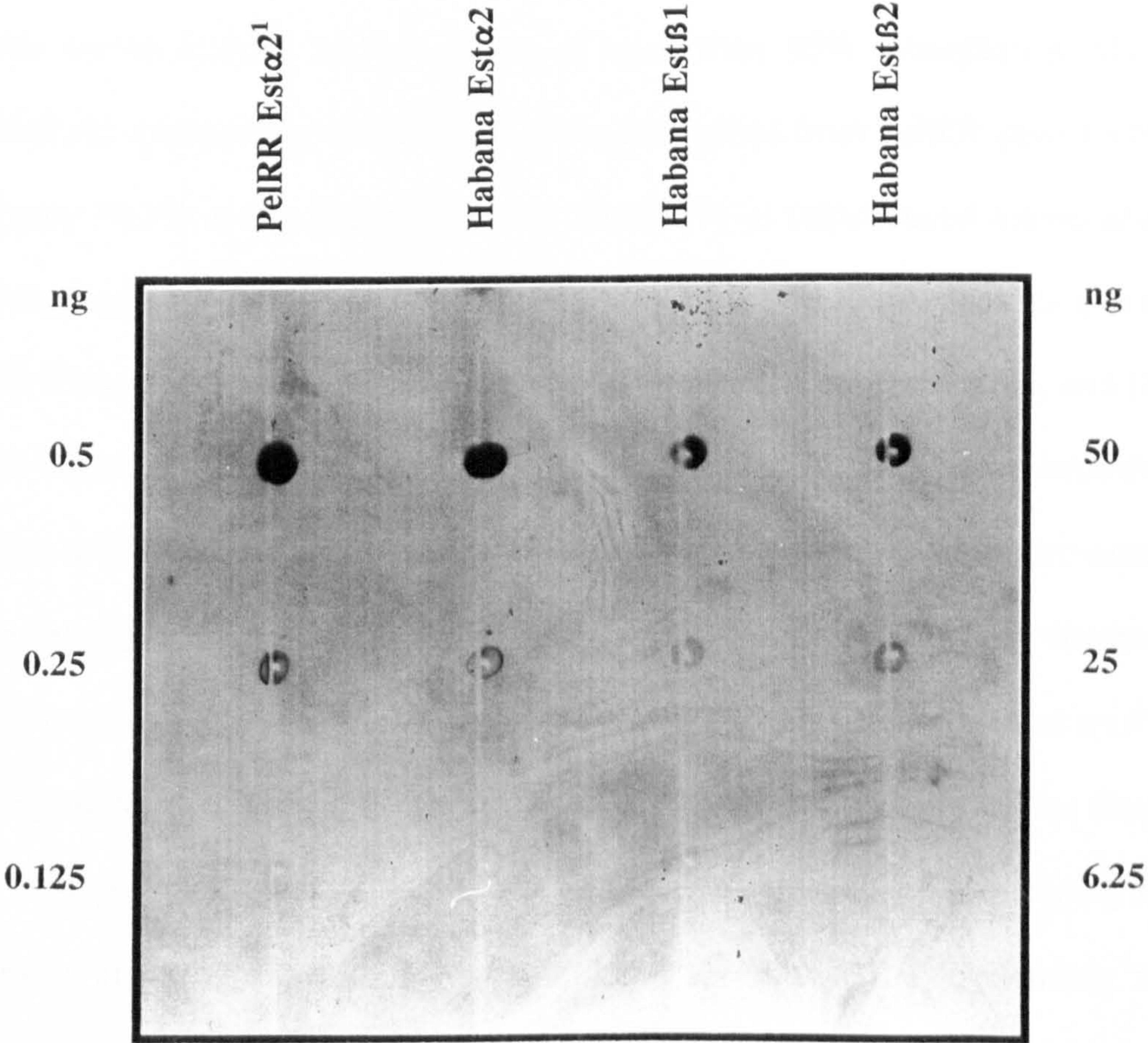
4.3.2. DOT-BLOT ASSAY

Cross-reactivity of the PelRR Est α 2¹ antiserum with purified Est α 2, Est β 1 and Est β 2 from the Habana strain was examined using the dot-blot technique combined

with the ECL detection system (FIGURE 4.2). Different protein concentrations of the PelRR Est α 2¹ were included on the dot-blot for comparison of cross-reactivity. The immunological affinity of the Habana Est α 2 appeared to be the same as that of the PelRR Est α 2¹ itself. The antiserum also cross-reacted with both the Habana Est β 1 and Est β 2, but this cross-reactivity was approximately 150-fold less than was observed for the Est α 2s. Cross-reactivity was also reported for the antiserum with PelRR Est β 2¹ (Karunaratne, 1994; Karunaratne *et al.*, 1995b), this being 50-fold less than with the Est α 2¹. These observations contrast with those of Mouches *et al.* (1987) who found no cross-reaction between an antiserum raised against denatured Est α 1 purified from the S54 strain of *C. pipiens* and Est β 1, Est β 2 and Est β 3 in strains of the *Culex* genus, and also contrast with the results of western-blots in this study (see section 4.3.1.). The most likely explanation for these contrasting observations lies in the different methods of detection, the ECL method being much more sensitive than either the 4-chloro-1-naphthol or 3,3'-diaminobenzidine tetrahydrochloride methods. The difference between the cross-reactivity of the PelRR Est β 2¹ and the Habana Est β 2 is strikingly large. No sequence data is available for the Habana *est* β 2 gene and it is not, therefore, known if its deduced amino acid sequence differs from that of the *est* β 2¹ gene. However, if the hypothesis of Raymond *et al.* (1991) is correct, and on the basis of restriction fragment data it is (Raymond *et al.*, 1991; Karunaratne *et al.*, 1995a), the expressed proteins of the two genes should be identical. Unfortunately, purified PelRR Est β 2¹ was not available for direct comparison alongside the Habana Est β 2 on a dot-blot in order to determine if the difference in cross-reactivity would be maintained under experimentally identical conditions.

FIGURE 4.2

Dot-blots of serial dilutions of esterases Est α 2, Est β 1 and Est β 2 purified from the Habana strain immunodetected with the PelRR Est α 2¹ antiserum using the ECL detection system. A serial dilution of the PelRR esterase Est α 2¹ was included for comparison.



This study, together with those of Mouches *et al.* (1987), Poirie *et al.* (1992) and Karunaratne *et al.* (1995b), has shown that the esterases α and β are immunologically distinct. There is also evidence that these two esterase types are distinct at the nucleotide and deduced amino acid levels. Comparison of the *est* β 1¹ gene (from the TEM-R strain (Mouches *et al.*, 1990)), the *est* β 1² gene (from the MRES strain), the *est* β 2¹ gene (from the PelRR strain), the non-amplified *est* β 1³ (from the susceptible PelSS strain), and a partial-length cDNA of an *est* β 2 gene (from the SeLax strain (deduced amino acid sequence only) (Raymond *et al.*, 1991)) have shown identity at both levels of more than 95% (Vaughan *et al.*, 1995). However, comparison of the *est* α 2¹ and *est* β 2¹ genes from PelRR gave a similarity of only 58.3% at the nucleotide level and 49.2% at the deduced amino acid level (Vaughan, 1995; Vaughan & Hemingway, 1995). It is probable that the esterases α and β arose through gene duplication from a common ancestral gene, and that the two esterase types now form distinct multi-allelic families. This hypothesis is supported by inheritance patterns which have shown that the resistance-associated esterase genes of the *Culex pipiens* complex are clustered on a single chromosome (chromosome III) (Pasteur *et al.*, 1981a), with the amplified *est* α and *est* β genes being under the control of two tightly linked loci (Pasteur *et al.*, 1981a; Pasteur *et al.*, 1981b; Poirie *et al.*, 1992). It has been suggested that the loci coding for the various elevated esterases α are homologous, and that the loci coding for the various elevated esterases β are also homologous (Poirie *et al.*, 1992).

Using the antiserum raised against the TEM-R Est β 1¹ (Mouches *et al.*, 1987) and the nitroblue tetrazolium salt detection method, a dot-blot assay has been

developed that has enabled the presence of elevated esterases β associated with organophosphate resistance to be diagnosed in field collections of *C. quinquefasciatus*, *C. pipiens* and *C. tarsalis* (Beyssat-Arnaouty *et al.*, 1989), and it was suggested that the same assay could be used with the antiserum raised against the S54 Est α 1 (Mouches *et al.*, 1987) to detect elevated esterases α . Using this method the reaction of the Est β 1¹ antiserum with AChE was apparently insignificant. Similarly, the immunoglobulin G (IgG) fraction of an antiserum raised against the aphid esterase E4, combined with an immunoplate assay based on measuring the esterases activity of E4 bound to IgG (Devonshire *et al.*, 1986a), was used to monitor frequencies of insecticide resistance (OP and pyrethroid resistance) in aphids in the field (French-Constant & Devonshire, 1988). Indeed, this immunoassay technique was found to be more sensitive in discriminating variants with different degrees of E4 elevation than the assaying of esterase activity (Devonshire *et al.*, 1992). Although the Est α 2¹ antiserum was seen to cross-react strongly with AChE using both the 3,3'-diaminobenzidine tetrahydrochloride dye method (in this study) and the ECL method (Karunaratne, 1994; Karunaratne *et al.*, 1995b) for detection, it is possible that this antiserum could be used for monitoring the presence of elevated esterases α associated with OP resistance in the same way. It has also been suggested that it may be of use in molecular biological studies for the screening of expression libraries for esterases of other organisms (Karunaratne *et al.*, 1995b). This suggestion was prompted by the observation that this antiserum cross-reacts with some vertebrate esterases and cholinesterases (Karunaratne, 1994; Karunaratne *et al.*, 1995b). However, it is unlikely to be of use in the cloning of other insect esterases as, although it was shown to have a moderate cross-reactivity

with other insect AChEs, it had no detectable activity with the esterases associated with resistance in planthoppers, cockroaches and grain beetles. The exception is possibly the malathion carboxylesterase of *Anopheles*, which gave a moderate cross-reaction with the Est α 2¹ antiserum (Karunaratne *et al.*, 1995b).

4.3. SUMMARY

Western-blot of crude homogenates of individuals of the Habana strain containing elevated esterases Est α 2, Est α 3, Est β 1, Est β 2 and other, as yet unclassified, esterases α and β were cross-reacted with the polyclonal antiserum raised against the PelRR Est α 2¹ and the results detected using the 3,3'-diaminobenzidine tetrahydrochloride dye method. Using this method the Est α 2¹ was observed to have a similar cross-reactivity with the Habana Est α 2 and Est α 3 as with the PelRR Est α 2¹ itself, but not to cross-react with any of the esterases β . The Est α 2¹ antiserum also cross-reacted strongly with the *C. quinquefasciatus* AChE showing that Est α 2¹ and AChE are immunologically closely related. A number of non-elevated esterases were observed to cross-react weakly with the antiserum. Dot-blot of the purified Est α 2, Est β 1 and Est β 2 from the Habana strain were also cross-reacted with the antiserum and the results detected using the highly sensitive ECL method. The Habana Est α 2 was observed to have a similar immunological affinity as the purified PelRR Est α 2¹ itself. The Habana Est β 1 and Est β 2 were also observed to cross-react with the antiserum but with a reactivity that was approximately 150-fold less than with the Est α 2s. The findings of this and previous

studies are discussed in terms of the possible evolutionary origin of the esterases α and β .

CHAPTER 5

KINETIC AND MOLECULAR CHARACTERISTICS OF Est β 1 IN STRAINS OF *C. QUINQUEFASCIATUS* FROM COLOMBIA AND TRINIDAD

5.1. INTRODUCTION

In contrast to the restriction fragment patterns for the amplified *est* β 2 genes in *C. quinquefasciatus* strains of different geographic origin, which were found to be identical (Raymond *et al.*, 1991; Karunaratne *et al.*, 1995a), the restriction fragment patterns for two amplified *est* β 1 genes, *est* β 1¹ in the Californian TEM-R strain (Mouches *et al.*, 1990) and *est* β 1² in the Cuban MRES strain, were found to differ (Vaughan *et al.*, 1995). Moreover, on sequencing these two *est* β 1 cDNAs, they were found to differ at both the nucleotide and amino acid levels, homologies being 95.2 % and 96.1% respectively (Vaughan *et al.*, 1995). On the basis of these data it was hypothesised that the genes coding for the two electrophoretically identical esterase Est β 1s from California and Cuba were allelic variants amplified independently (Vaughan *et al.*, 1995). This differs from the hypothesis for elevated Est β 2, namely that this arose from the a single amplification of an *est* β 2 gene that has subsequently spread worldwide by migration (Raymond *et al.*, 1991). The present study details the restriction fragment analysis of *est* β 1, and the kinetic characterization of Est β 1, present in two *C. quinquefasciatus* strains originating from Colombia and Trinidad.

5.2. MATERIALS AND METHODS

5.2.1. MOSQUITO STRAINS

The Colombia and Trinidad strains were collected from Nuqui-Choco, Colombia and Port of Spain, Trinidad respectively in 1993. The population from which the Colombia strain originated was not under any apparent insecticidal selection pressure. The field population of Trinidad was exposed as larvae to temephos and as adults to ultra-low volume spraying of malathion. Both Colombia and Trinidad strains were maintained in the laboratory without insecticide selection. Conditions and methods for colony maintenance were as for the Habana strain (see section 2.2.2.).

5.2.2. CHEMICALS AND EQUIPMENT

The Nick spin columns were purchased from Pharmacia, U.K. All other chemicals and equipment required for the insecticide bioassays, for the purification of the esterase EstB1s, and for their characterisation were the same as detailed for the Habana EstB1 in section 2.2.3.

5.2.3. LARVAL BIOASSAYS

Larval bioassays were carried out and the results analyzed as in section 2.2.4. The insecticides used were malathion (phosphorothionate), propoxur (carbamate) and

lambda-cyhalothrin (pyrethroid).

5.2.4. BIOCHEMICAL ASSAYS FOR THE DETECTION OF THE ALTERED ACETYLCHOLINESTERASE AND GLUTATHIONE S-TRANSFERASE INSECTICIDE RESISTANCE MECHANISMS

The biochemical assay techniques for detection of altered AChE and elevated glutathione S-transferase-based insecticide resistance mechanisms, and the assay for protein concentration, were carried out as described in section 2.2.5.

5.2.5. RESTRICTION FRAGMENT ANALYSIS OF GENOMIC DNA

DNA was extracted by an adaptation of the method of Miller *et al.* (1988). About 1 gm wet weight of fourth instar larvae were ground in liquid nitrogen. The homogenate was added to 10 vol. of extraction buffer [10 mM Tris/HCL (pH 8)/0.1 M EDTA (pH 8)/0.5% (w/v) SDS/20 µg/ml pancreatic RNAase]. After incubation for 1 hr at 37 °C, proteinase K was added to a final concentration of 100 µg/ml and the homogenate was incubated at 50 °C for 3 hr. After cooling on ice for 10 min, 0.35 vol. of saturated NaCl was added to precipitate protein. The homogenate was well mixed and stored on ice for 5 min. then centrifuged at 16,000 g for 20 min. The supernatant was removed and the DNA precipitated by adding an equal volume of propan-2-ol. The DNA was re-suspended in 7.5 ml of Tris EDTA buffer (TE) (pH 8) containing 20 µg/ml RNAase and incubated at 37 °C for 1 hr, then extracted with phenol/chloroform and finally chloroform. After precipitation with ethanol, the

DNA was re-suspended in a small volume of TE and stored at 4 °C until used for Southern blotting.

A PelRR *estB2*¹ cDNA fragment (Vaughan *et al.*, 1995) was used as a probe to determine the haplotype of the esterase. Aliquots of genomic DNA (10 and 15 µg for the Trinidad strain; 1, 2, 3, 4, and 5 µg for the Colombia strain) were digested to completion with *Eco*RI and separated by gel electrophoresis through 0.8% w/v agarose. The DNA was transferred to charged nylon membranes and hybridised with the ³²P-labelled *estB2*¹ probe (sp. activity > 2 x 10⁶ c.p.m./µg) at 65 °C for 16 hr in hybridisation buffer [5 x Denhardts solution/ 6 x SSC (1 x SSC is 0.15 M NaCl/0.015 M sodium citrate)/0.1% (w/v) SDS/0.1% (w/v) sodium pyrophosphate/5% (w/v) polyethylene glycol 8000/100 µg/ml boiled herring sperm DNA]. The final membrane washes were at 65 °C in 0.1 x SSC and 0.1% (w/v) SDS for 20 min. The membrane was then exposed to Kodak X-OMAT film.

5.2.6. PURIFICATION AND CHARACTERISATION OF *EstB1* FROM THE COLOMBIA AND TRINIDAD STRAINS

Purification of esterase *EstB1* from the Colombia and Trinidad strains was achieved by use of the four chromatography steps (Q-Sepharose, phenyl-Sepharose, hydroxyapatite and *p*-chloromercuribenzoate-agarose) under the conditions previously described for purification of the equivalent esterase from the Habana strain (see section 2.2.8.). On running the decreasing salt gradient for elution of proteins from the phenyl-Sepharose column, two activity peaks were observed. This

esterase was found to be elevated Est α 3 (see section 5.3.3. for further details). Esterase Est β 1 was obtained from three purifications using larvae from three different generations for use in subsequent characterizations to ensure that all results were reproducible.

Bimolecular rate constants, k_2 s, were determined as described in section 2.2.9.4.2. The rate of deacylation of Colombia Est β 1 was also determined. In the reactivation experiments, enzyme was incubated with the respective insecticide for 10-15 min so that the enzyme was more than 90% inhibited. The concentration of the enzyme was determined as described previously (see section 2.2.6.). The unbound insecticide and enzyme-insecticide complex were separated on a Nick spin column following the manufacturers methodology. At set time intervals 10 μ l aliquots of the reactivating enzyme were removed and esterase activity measured. The percentage activity was plotted against time and the slope of the regression line through data points used to obtain the reactivation constant, k_3 .

5.3. RESULTS AND DISCUSSION

5.3.1. LARVAL BIOASSAYS

The bioassay of fourth instar Trinidad larvae with malathion (phosphorothionate), propoxur (carbamate) and lambda-cyhalothrin (pyrethroid) indicates that there is a low level of resistance to these insecticides in this strain (TABLE 5.1 and FIGURES 5.1-5.3). For lambda-cyhalothrin the difference between the LC50s of the

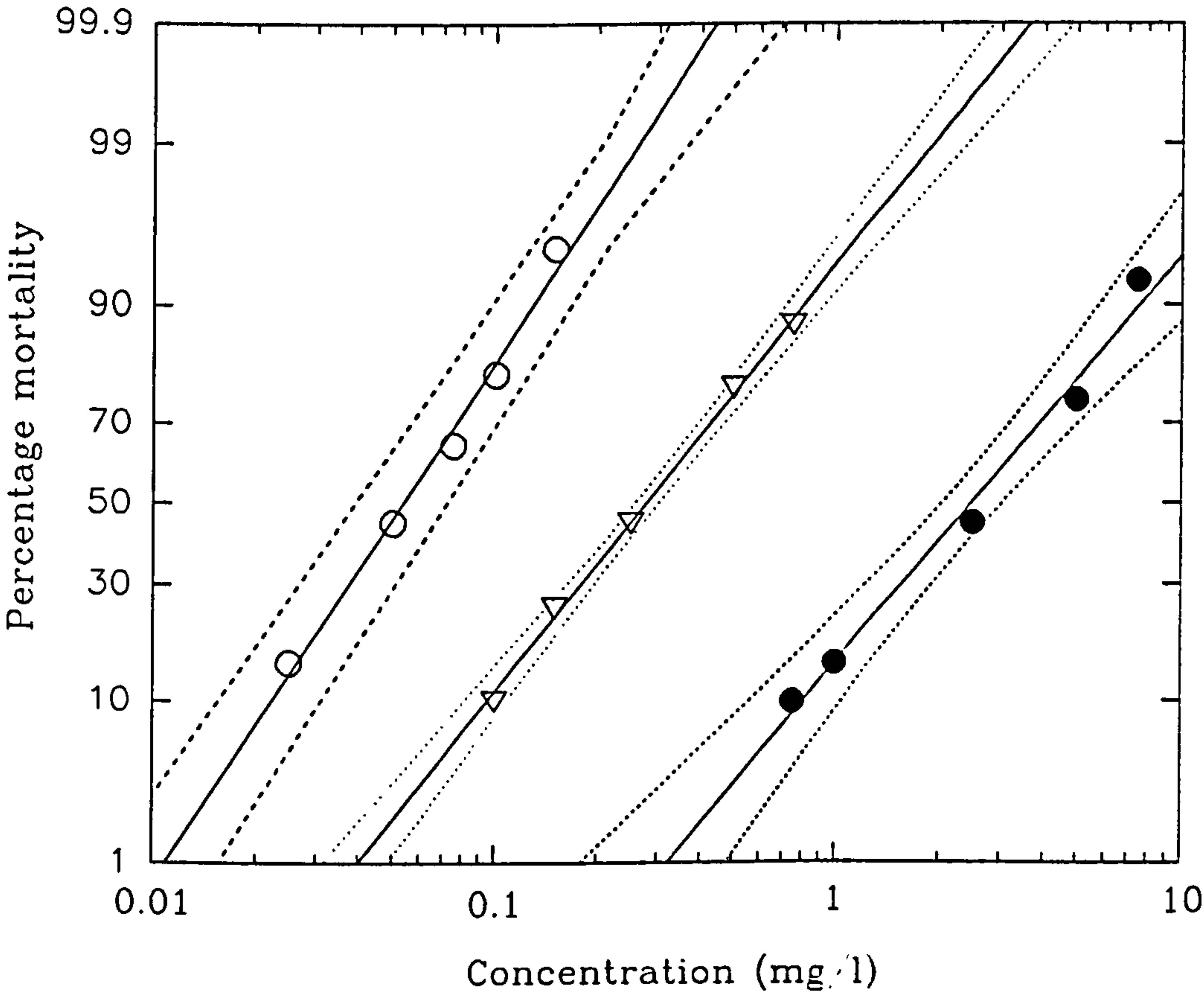
TABLE 5.1. Log-dosage probit mortality regression analysis for malathion (organophosphorus), propoxur (carbamate) and lambda-cyhalothrin (pyrethroid) insecticides applied to fourth instar larvae of the field-collected Colombia and Trinidad strains of *C. quinquefasciatus*. The resistance ratios (RR) for both strains were calculated using LC₅₀ and LC₉₀ values obtained for the susceptible PelSS strain. Slopes of the probit lines are given as an indication of the relative homogeneity of data. No regression data had Chi-square values which deviated significantly from a straight line ($P = <0.05$). Upper (U.L.) and lower (L.L.) 95% confidence limits are attached to each

LC₅₀.

Insecticide	Mosquito Strain				Trinidad							
	Colombia											
	LC ₅₀	LC ₉₀	Slope		LC ₅₀	LC ₉₀	Slope					
	mg/l	RR	mg/l	RR	mg/l	RR	mg/l	RR				
Malathion	2.55 U.L. 3.08 L.L. 2.11	47.12	7.94	59.9	1.13	n=200	0.28 U.L. 0.33 L.L. 0.23	5.09	0.80	6.0	1.20	n=200
Propoxur	0.76 U.L. 0.91 L.L. 0.67	4.77	1.82	3.7	1.47	n=200	0.40 U.L. 0.50 L.L. 0.32	2.52	1.13	2.3	1.24	n=120
Lambda-cyhalothrin	0.0024 U.L. 0.0030 L.L. 0.0019	2.40	0.0063	2.3	1.31	n=100	0.0017 U.L. 0.0021 L.L. 0.0013	1.70	0.0047	1.7	1.25	n=100

FIGURE 5.1

Log-dosage mortality line for malathion in the field-collected Colombia and Trinidad strains compared to the standard susceptible PelSS strain.



○ PelSS, 95% confidence limits
▽ Trinidad, 95% confidence limits
● Colombia, 95% confidence limits

FIGURE 5.2

Log-dosage mortality line for propoxur in the field-collected Colombia and Trinidad strains compared to the standard susceptible PelSS strain.

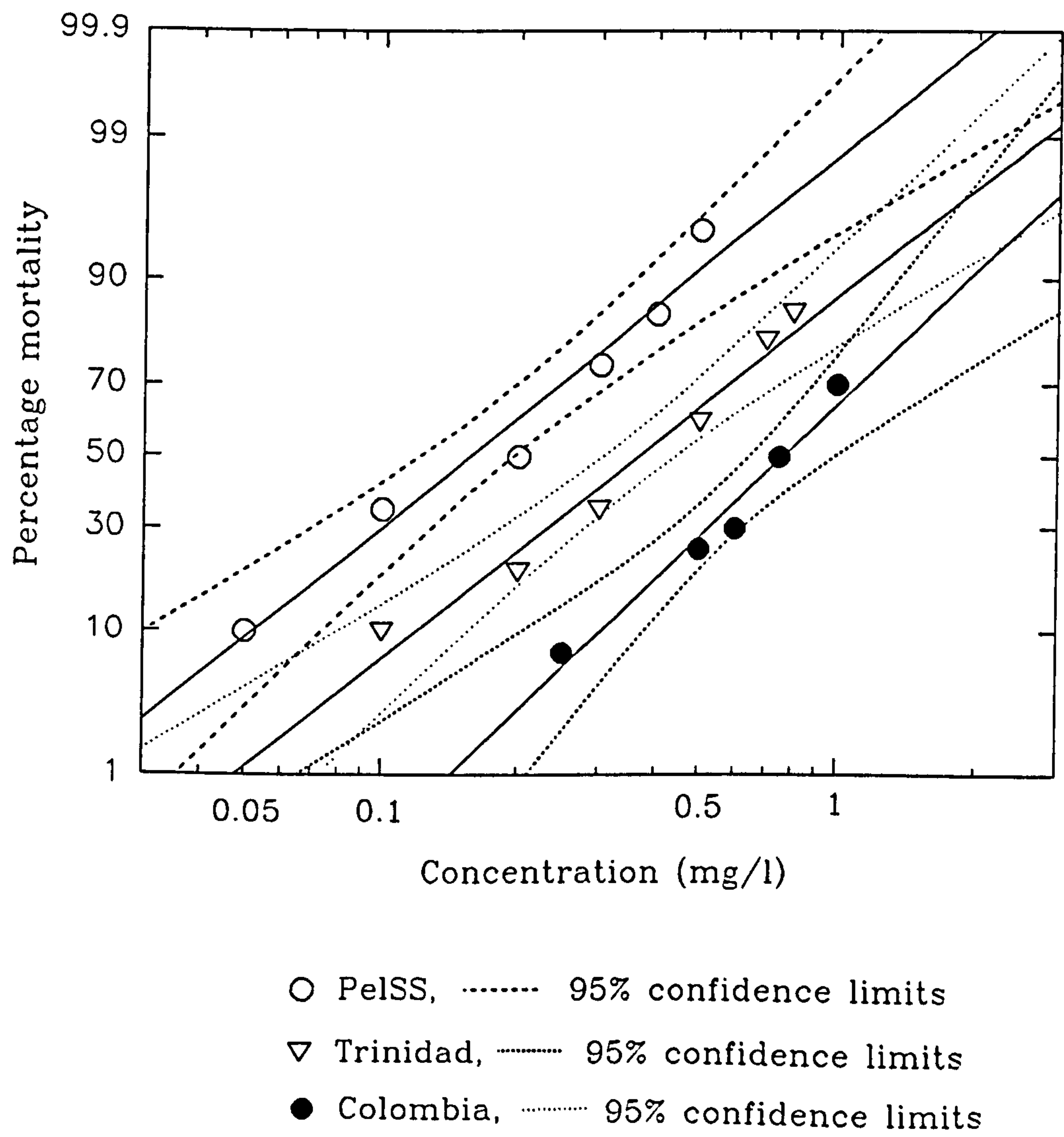
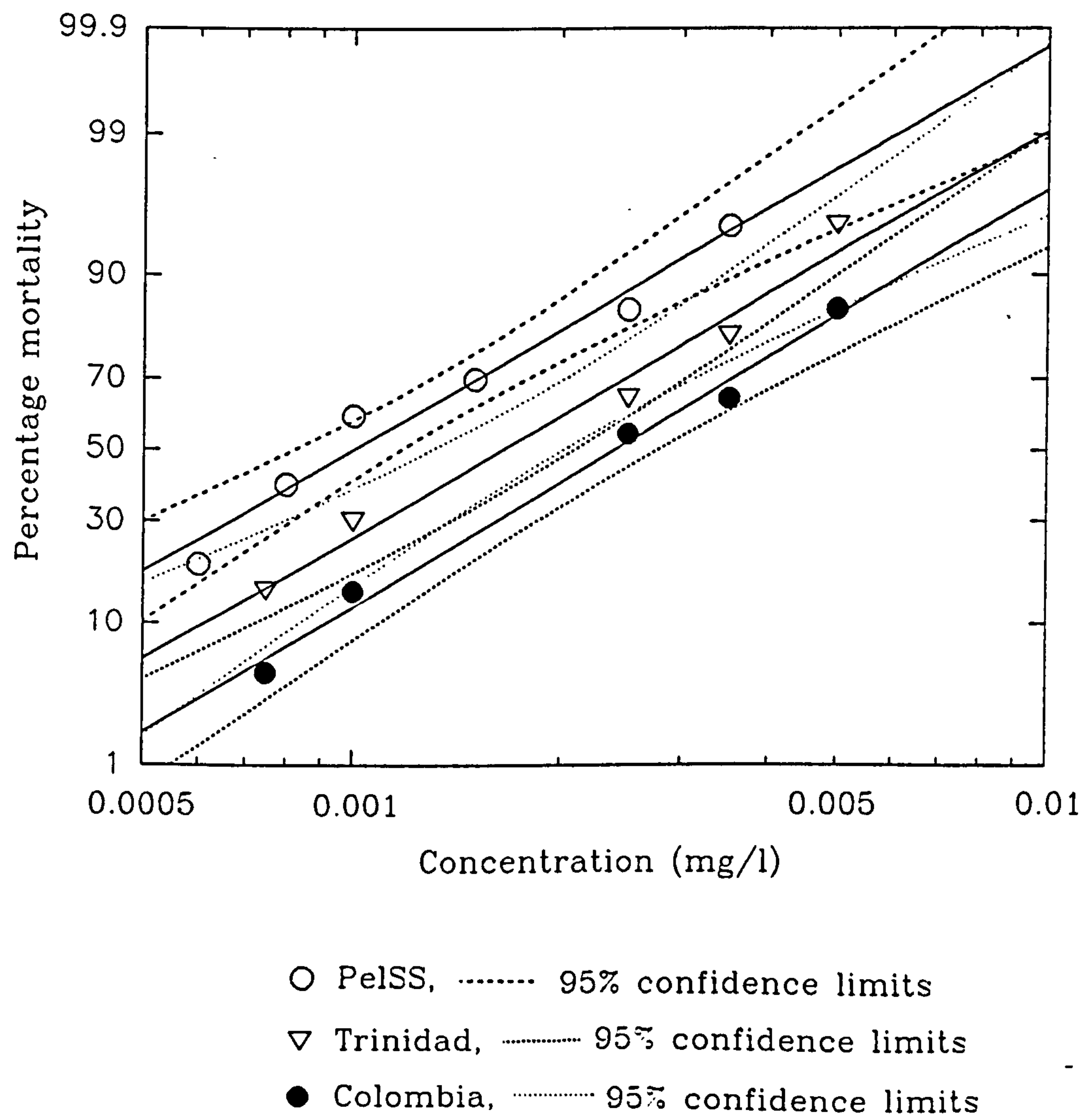


FIGURE 5.3

Log-dosage mortality line for lambda-cyhalothrin in the field-collected Colombia and Trinidad strains compared to the standard susceptible PelSS strain.



Trinidad and susceptible PelSS strains was statistically significant but at the LC90 the 95% confidence limits overlapped (FIGURE 5.3). For the bioassay of fourth instar Colombia larvae, both the LC50 and LC90 for these three insecticides was significantly higher than for the PelSS. However, the only insecticide to which resistance of the Colombia strain was high was malathion, resistance being low to both propoxur and lambda-cyhalothrin. There is no record of the population from which the Colombia strain was derived ever having been the subject of any control campaign. It is likely, therefore, that resistance in this strain is due to exposure to organophosphorus pesticides used in agriculture, possibly through contamination of water in larval habitats.

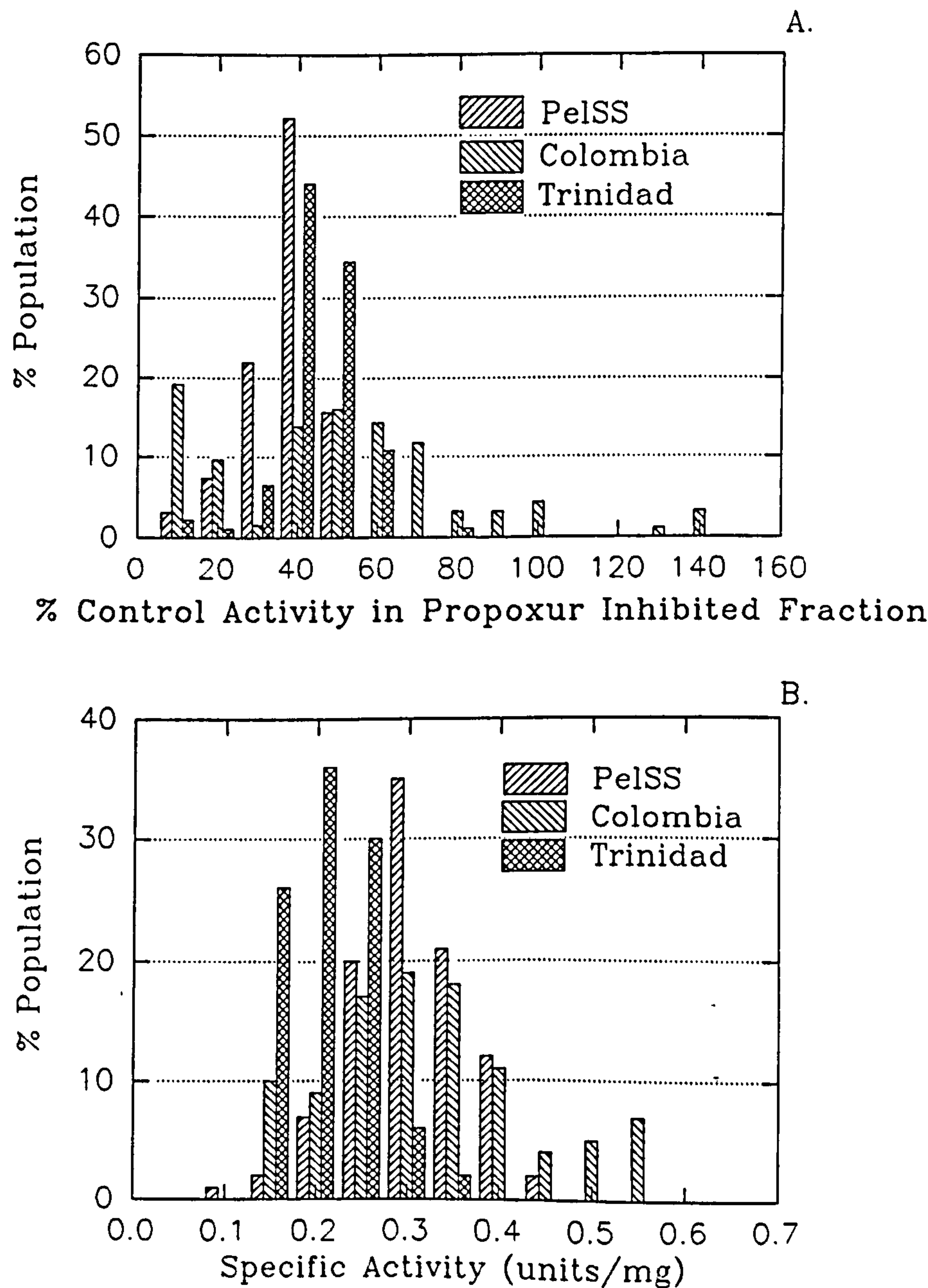
5.3.2. BIOCHEMICAL ASSAYS FOR THE DETECTION OF ALTERED ACETYLCHOLINESTERASE AND GLUTATHIONE S- TRANSFERASE INSECTICIDE RESISTANCE MECHANISMS

The biochemical assay of the Trinidad strain for the altered AChE-based mechanism of OP and carbamate resistance, as revealed by the AChE inhibition profile for this strain (FIGURE 5.4 A.), showed that this mechanism was not present. This suggests that the low level of resistance to malathion and propoxur is due to the elevated esterase-based mechanism. In contrast, the AChE inhibition profile for the Colombia strain (same figure) showed that 4 individuals were homozygous for the altered AChE and 7 individuals were in the heterozygous state from a total of 95 larvae assayed. The frequency of the altered AChE in the Colombia strain, calculated using the Hardy-Weinberg equation, was 0.08. The two

95 larvae of each of the three strains (PelSS, Colombia and Trinidad) were assayed for acetylcholinesterase inhibition and glutathione *S*-transferase activity

FIGURE 5.4

Acetylcholinesterase inhibition profiles (A) and glutathione *S*-transferase activity profiles (B) in fourth instar larvae of the Colombia and Trinidad strains. Data for the standard susceptible PelSS strain are presented for comparison. A unit is equivalent to the production of 1 μ mole of Glutathione-CDNB conjugate.



strains were not assayed for the elevated esterase-based mechanism. However, the higher degree of amplification of the *estβ1* gene (see section 5.3.2.), and higher yield of Estβ1 from purifications would suggest that the degree of elevation of Estβ1 is higher in the Colombia than in the Trinidad strain. This, together with the higher frequency of the altered AChE in the Colombia strain, explains the higher resistance to both malathion and propoxur when compared to the Trinidad strain.

The profiles for glutathione *S*-transferase GST activity (FIGURE 5.4 B.) show that, whilst the distribution of specific activities of Trinidad larvae is similar to that of the susceptible PelSS strain, the distribution of activities of Colombia larvae is different with some individuals having high activity. Increases in GST activity have been noted in *C. quinquefasciatus* as a mechanism of DDT resistance (Amin & Hemingway, 1989) and its association with resistance to this insecticide has also been inferred from increases in the metabolism of DDT to DDE in resistant strains (Khayrandish & Wood, 1993a). The Colombia strain was not tested for resistance to this insecticide. GSTs have also been found to be responsible for OP resistance, both by glutathione conjugation with the *O*-alkyl portion of the insecticide (*O*-dealkylation) and by conjugation with the *O*-aryl portion (Hayes & Wolf, 1988). Whilst GST-mediated *O*-dealkylation has been reported to be responsible for OP resistance in the housefly, *M. domestica* (Motoyama & Dauterman, 1972; Oppenoorth *et al.*, 1979), the sheep blowfly, *L. cuprina* (Kotze & Rose, 1987), and possibly the mosquito *An. subpictus* (linked with DDT resistance) (Hemingway *et al.*, 1991), this mechanism has not been found in *C. quinquefasciatus* and it is unlikely, therefore, that the increase in GST activity in the Colombia strain

contributed to the observed OP resistance.

5.3.3. RESTRICTION FRAGMENT ANALYSIS OF GENOMIC DNA

Genomic DNA was extracted from Colombia and Trinidad fourth instar larvae, digested with the restriction endonuclease *EcoRI* and electrophoresed through a 0.8% agarose gel. After transfer to a charged nylon membrane, the fragments of digested DNA were hybridised with a 1350 bp ³²P-labelled PelRR *estβ2¹* cDNA probe. The membrane was then washed at high stringency and fragments with which the probe had hybridised were detected on X-Ray film (FIGURE 5.5). The relative mobility of each standard (of a λDNA/*HindIII* fragment ladder and a 1 kb ladder) was plotted against log size (kilobases, kb) to obtain a regression line. The size of the DNA fragments with which the PelRR *estβ2¹* probe had hybridised were calculated from this line (FIGURE 5.6). The *EcoRI* banding pattern shows a doublet of bands from the Colombia strain at 4.6 and 2.0 kb. These bands are intense compared to other bands in the same lane suggesting that the *estβ1* gene in the Colombia strain is highly amplified. The doublet of bands shows that there is an *EcoRI* site within the DNA covered by the probe, the doublet being the two fragments of this length of DNA either side of the restriction site. A doublet of bands (at 3.2 and 3.0 kb) was also found in *EcoRI* digests of the MRES *estβ1* gene (Vaughan *et al.*, 1995). It was thought probable in this case, that this was caused through observed variation (either an insertion or a deletion) in the non-transcribed leader sequence of the *estβ1* gene, only one nucleotide sequence being found in the sequencing of cDNA (Vaughan *et al.*, 1995). However, it has yet to be

FIGURE 5.5

Southern-blot of genomic DNA of the Colombia and Trinidad strains digested with *EcoRI* and hybridised with a 1350 bp ³²P-labelled PelRR estβ2¹ cDNA probe. Lanes 2 and 3 are 15 and 10 μg of digested Trinidad genomic DNA; lanes 4, 5, 6, 7 and 8 are 5, 4, 3, 2 and 1 μg of digested Colombia genomic DNA respectively. Lanes 1 and 9 are a λDNA/*Hind*III fragment ladder and a 1Kb ladder.

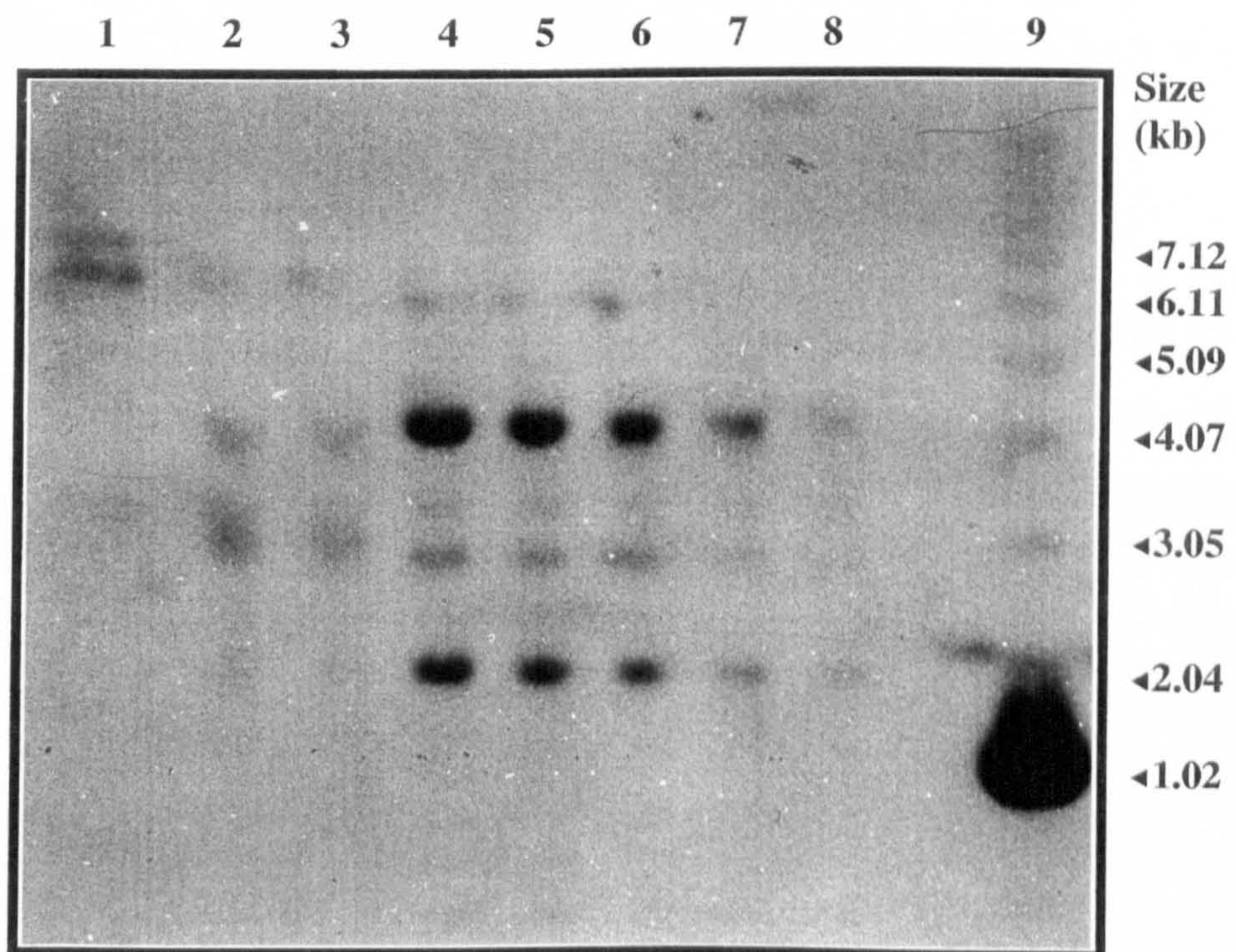
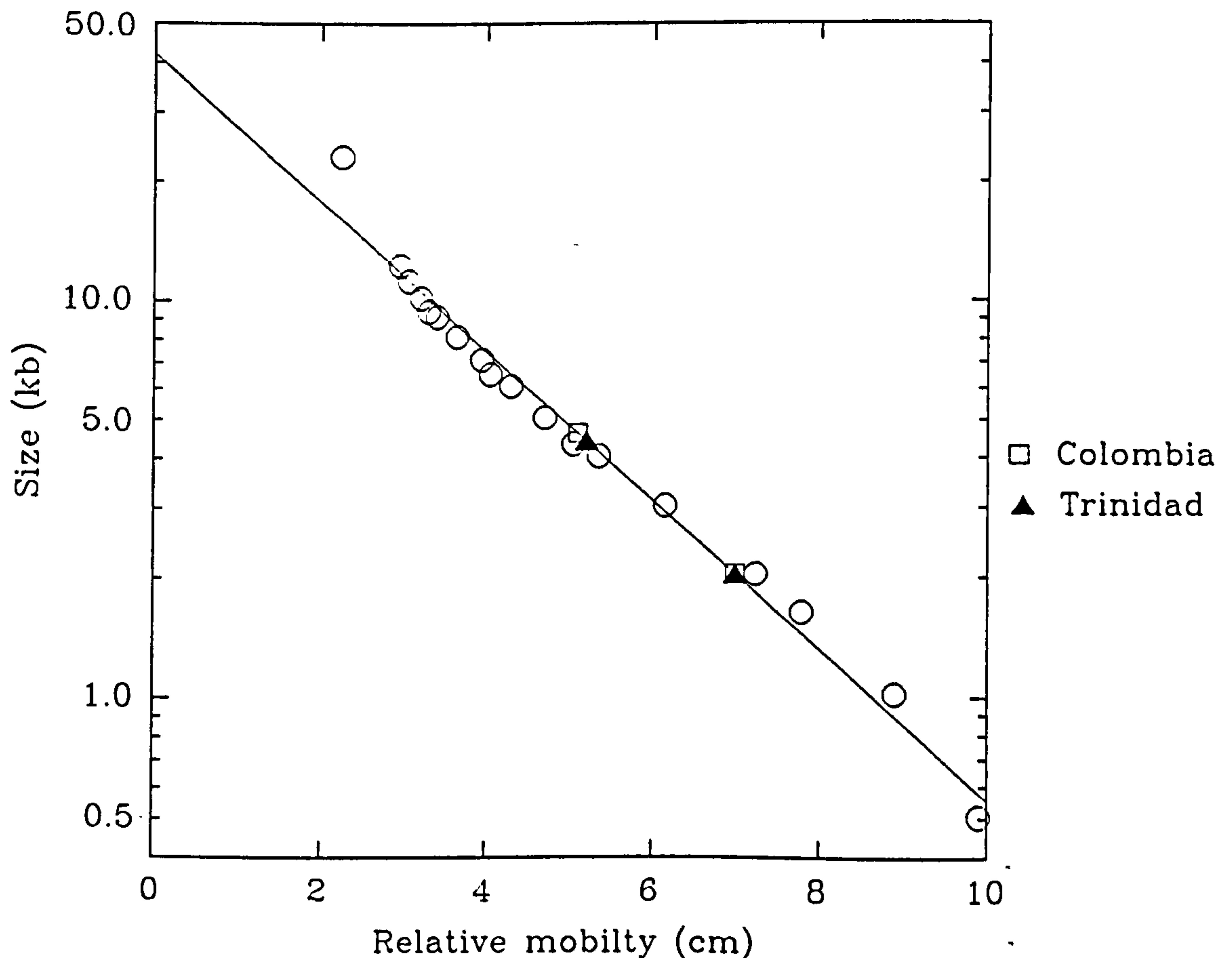


FIGURE 5.6

Estimation of fragment size (kilobases, kb) of restriction endonuclease *EcoRI* digested genomic DNA of the Colombia and Trinidad strains hybridised with a 1350 bp ^{32}P -labelled PelRR est $\beta 2^1$ cDNA on southern-blots. The relative mobilities of the standards (a λ DNA/*Hind*III fragment ladder and a 1kb ladder) were plotted against their size (on a log scale). The size of the major bands hybridising with the probe were estimated graphically for each blot.



shown that there is no *EcoRI* site in the introns of the gene. This can only be done by obtaining sequence from MRES genomic DNA. In relation to the Colombia bands those of the Trinidad strain are much less intense indicating a lesser degree of amplification. The upper of the doublet of bands in the Trinidad strain with a relative mobility close to that of the Colombia band is 4.4 Kb whilst the lower of these bands is 2.0 Kb. The estimates of restriction fragment size for the Colombia and Trinidad were obtained from digests of DNA of the two strains on the same blot and in adjacent lanes suggesting size differences were not due to errors in estimation. The *EcoRI* banding patterns of the two strains appear, therefore, to differ from each other. The *estB1*¹ gene in TEM-R has a 2.1 kB *EcoRI* RFLP (Raymond *et al.*, 1991), whilst the *estB1*² gene in the MRES strain has a doublet of 3.2 and 3.0 kB (Vaughan *et al.*, 1995). There are no bands of the same size as those for the Colombia and Trinidad *EstB1* in either TEM-R or MRES. Therefore, both the Colombia and Trinidad *estB1* genes differ in the size of their *EcoRI* restriction fragments with the two previously analyzed strains. To determine if the Colombia and Trinidad *estB1* genes differ from those in the TEM-R and MRES strains a full restriction map of the *estB1* genes should be completed. In addition, to determine whether the *EcoRI* site creating the doublet of bands in the two strains lies within the *estB1* gene (either in the coding sequence or the non-transcribed introns) or in the flanking regions of the gene, digested DNA can be hybridised with probes specific to the 5' and 3' ends of the gene. If the *EcoRI* site is internal to the gene, hybridisation with either probe should give only one band. However, if the *EcoRI* is external to the gene the doublet of bands will still be seen.

The bands of lower intensity in the Colombia and Trinidad strains are probably non-amplified *est* β genes. Comparing the pattern of these bands in the two strains it can be seen that none are of the same size. Whilst this is not proof that the non-amplified esterases are different in the two strains, Raymond *et al.* (1991) have reported a high level of polymorphism of the non-amplified *est* β locus and its flanking regions in different populations of *C. pipiens*, and in single mosquitoes from within a population.

5.3.4. PURIFICATION AND CHARACTERISATION OF *Est* β 1 FROM THE COLOMBIA AND TRINIDAD STRAINS

Using the four chromatography steps (Q-Sepharose, phenyl-Sepharose, hydroxyapatite and *p*-chloromeruribenzoate-agarose) described in section 2.2.8. the Colombia and Trinidad *Est* β 1s were separated from all contaminating proteins. Chromatography profiles for the purification of these esterases are shown in FIGURES 5.7-5.12. For the elution profiles of both the Colombia and Trinidad esterases purifications on phenyl-Sepharose (FIGURES 5.8 and 5.11), two activity peaks can be seen. On running aliquots of fractions lying within these peaks on a native PAGE gel stained for activity, the first peak was found to be an *Est* α of the same mobility as the *Est* β 1 in the second peak. This *Est* α was not visible on native PAGE of homogenates of individuals of this strain, it being masked by the colour produced from activity of the elevated *Est* β 1 which has a similar relative mobility. After comparing the relative mobility of this *Est* α with *Est* α s found in other strains of *C. quinquefasciatus* and *C. pipiens* (see Villani *et al.*, 1983; Pasteur & Georgiou,

FIGURE 5.7

Elution profile of Q-Sepharose chromatography for esterases Est α 3 and Est β 1 from the Colombia strain.

5 ml fractions of the elution of 0-0.5 M NaCl gradient in 25 mM Bistris propane (pH 7.0) were collected.

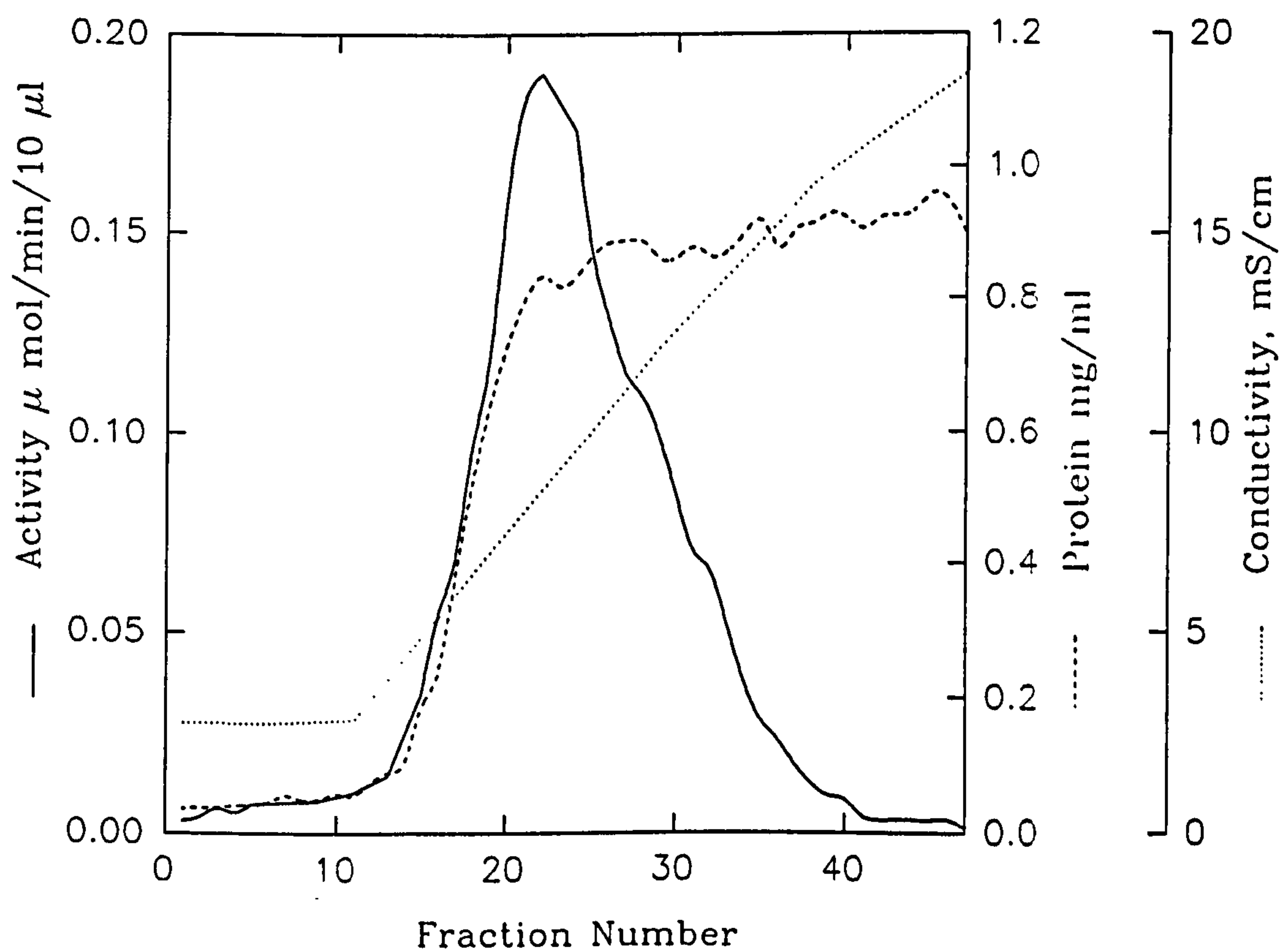


FIGURE 5.8

Elution profile of phenyl-Sepharose chromatography for esterases Est α 3 and Est β 1 from the Colombia strain.

5ml fractions of the elution of 3-0 M NaCl gradient in 25 mM Bistris propane buffer (pH 6.5), followed by isocratic elution with the same buffer, were collected. Esterase Est α 3 eluted in a separate peak to esterase Est β 1.

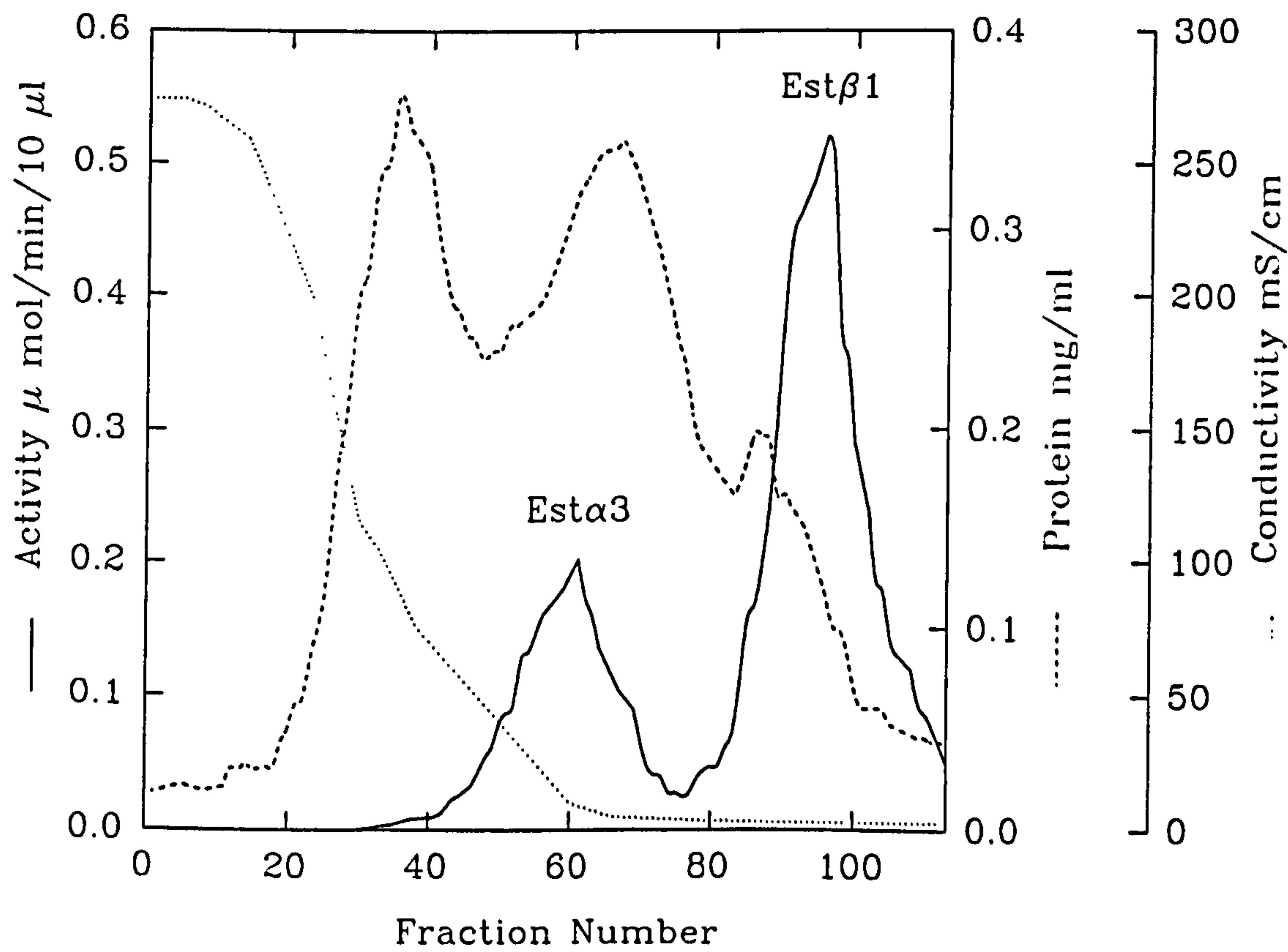


FIGURE 5.9

Elution profile of hydroxyapatite chromatography for esterase Est β 1 from the Colombia strain.

1 ml fractions of the elution were collected. The sample containing the esterases was applied to the column in 10 mM phosphate buffer (pH 6.8) with 50 mM NaCl and eluted with a 10-200 mM phosphate buffer gradient without NaCl.

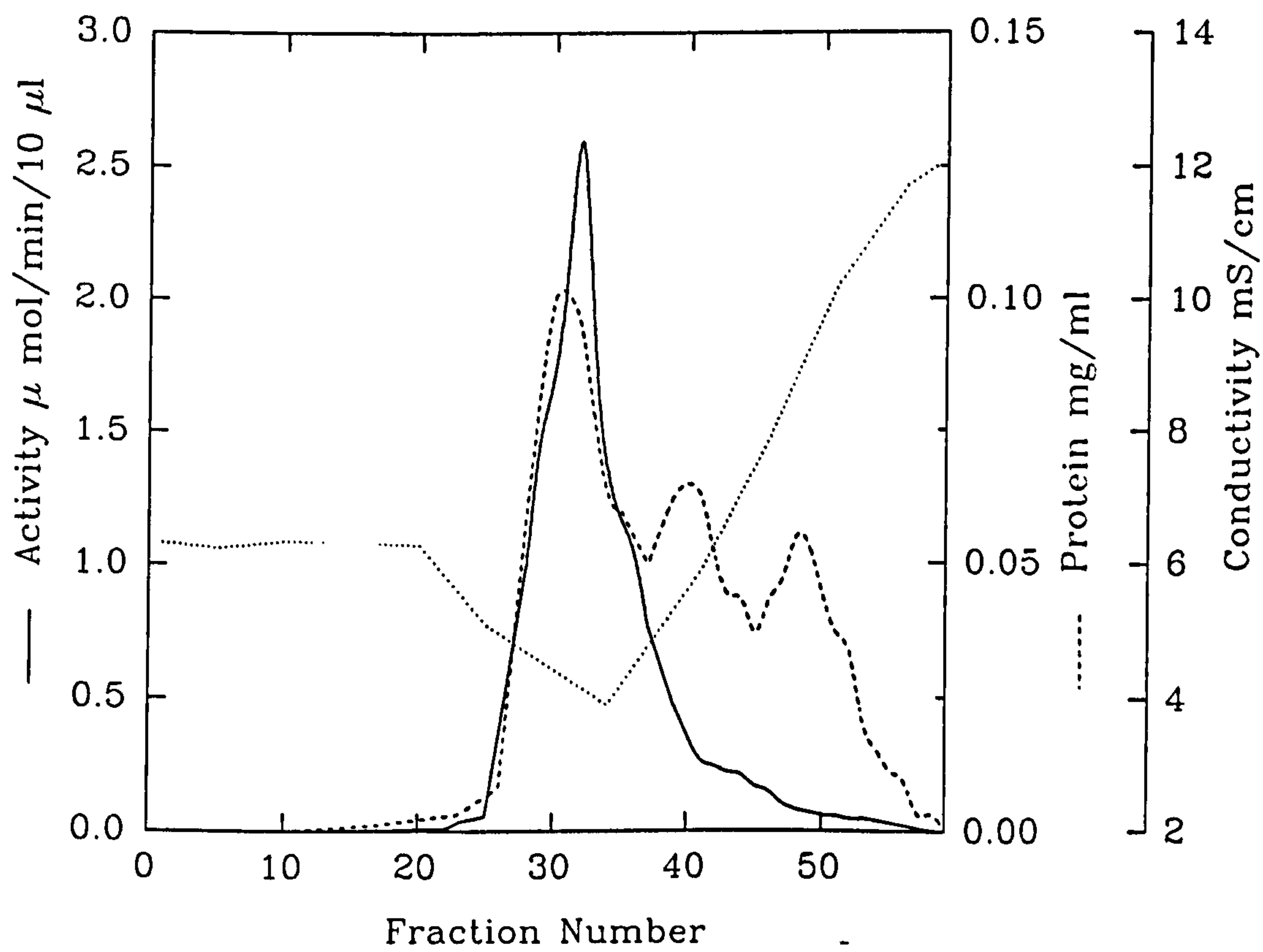


FIGURE 5.10

Elution profile of Q-Sepharose chromatography for esterases Est α 3 and Est β 1 from the Trinidad strain.

5 ml fractions of the elution of 0-0.5 M NaCl gradient in 25 mM Bistris propane (pH 7.0) were collected.

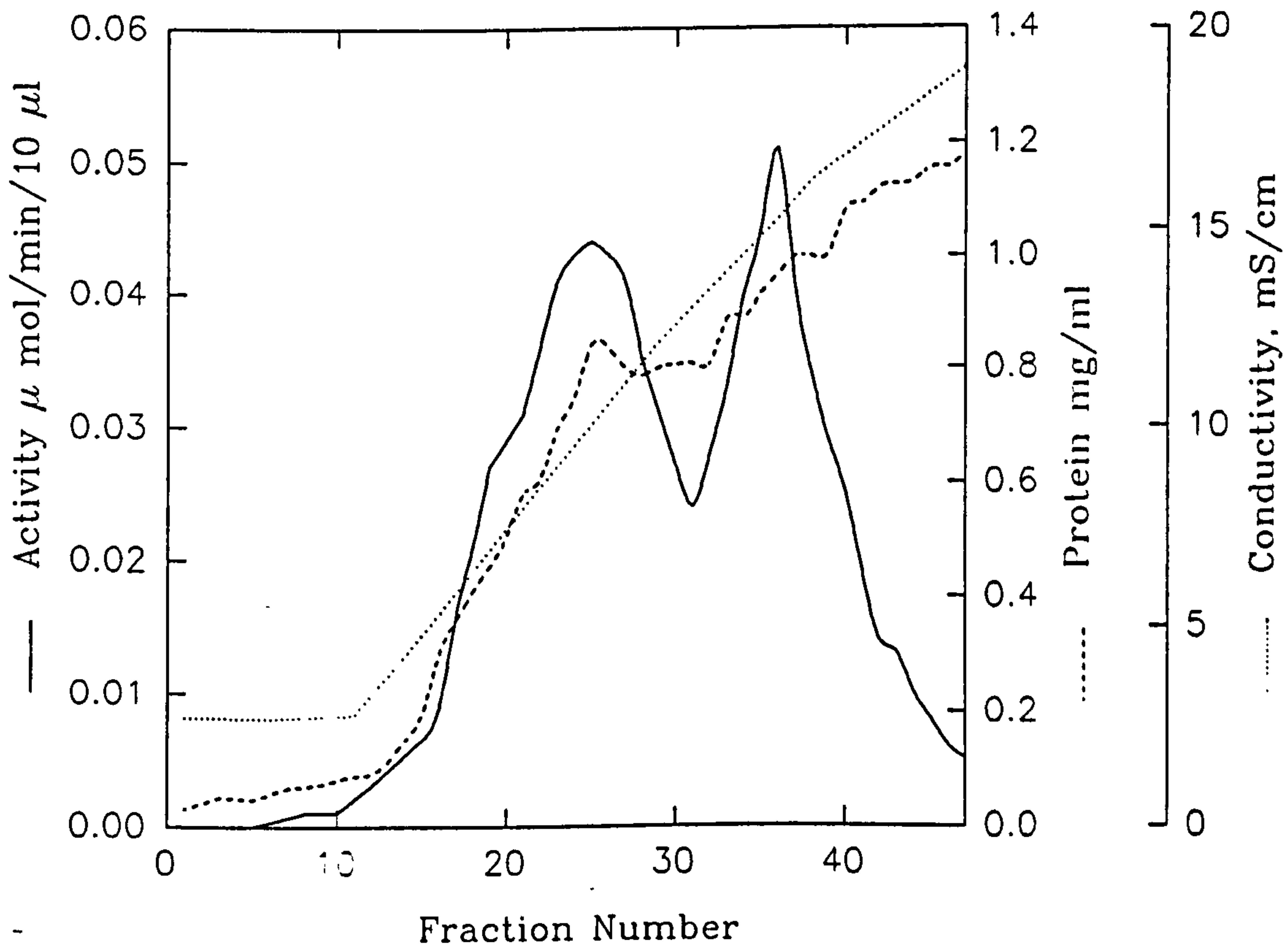


FIGURE 5.11

Elution profile of phenyl-Sepharose chromatography for esterases Est α 3 and Est β 1 from the Trinidad strain.

5ml fractions of the elution of 3.0 M NaCl gradient in 25 mM Bistris propane buffer (pH 6.5), followed by isocratic elution with the same buffer, were collected. Esterase Est α 3 eluted in a separate peak to esterase Est β 1.

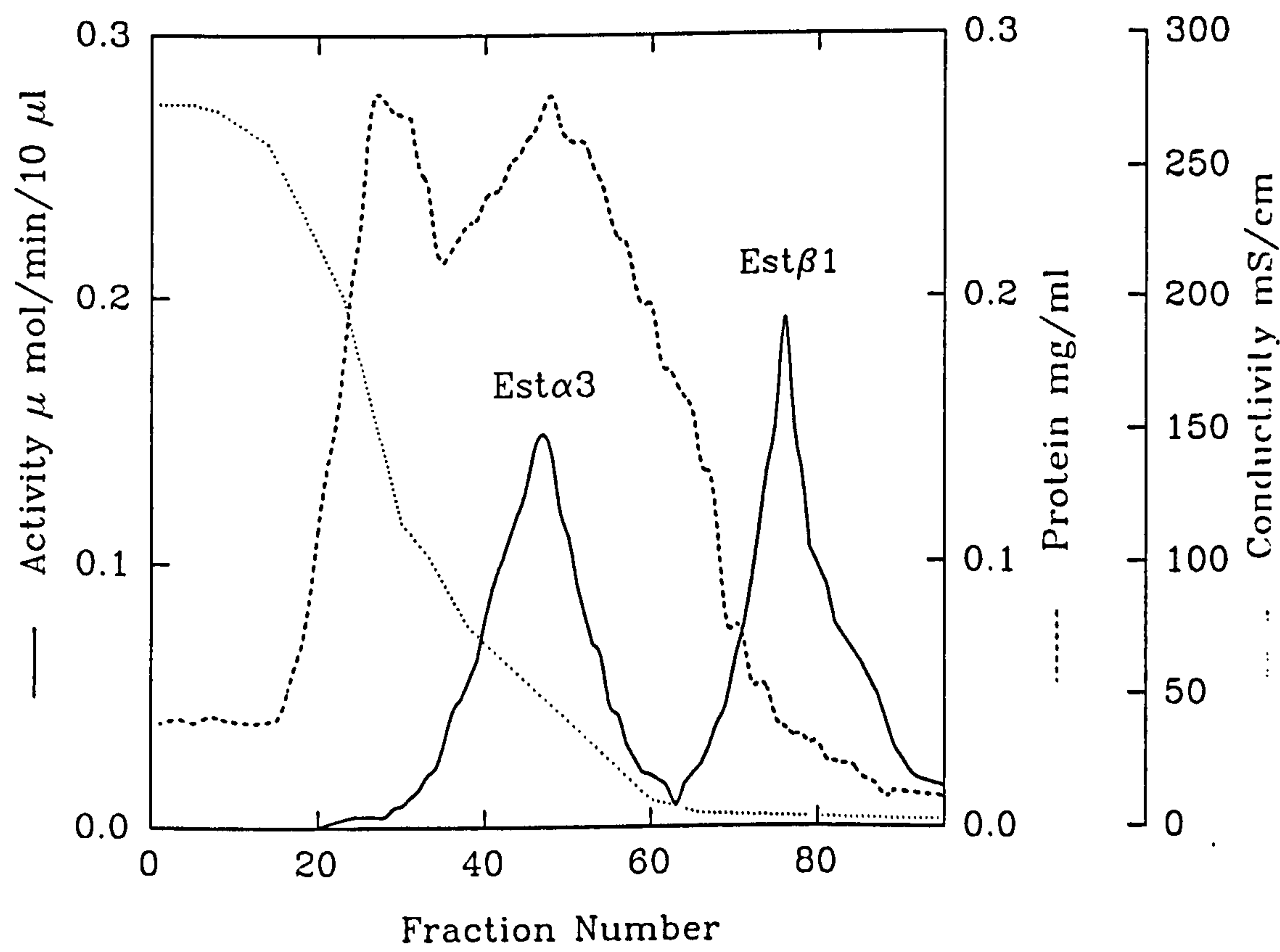
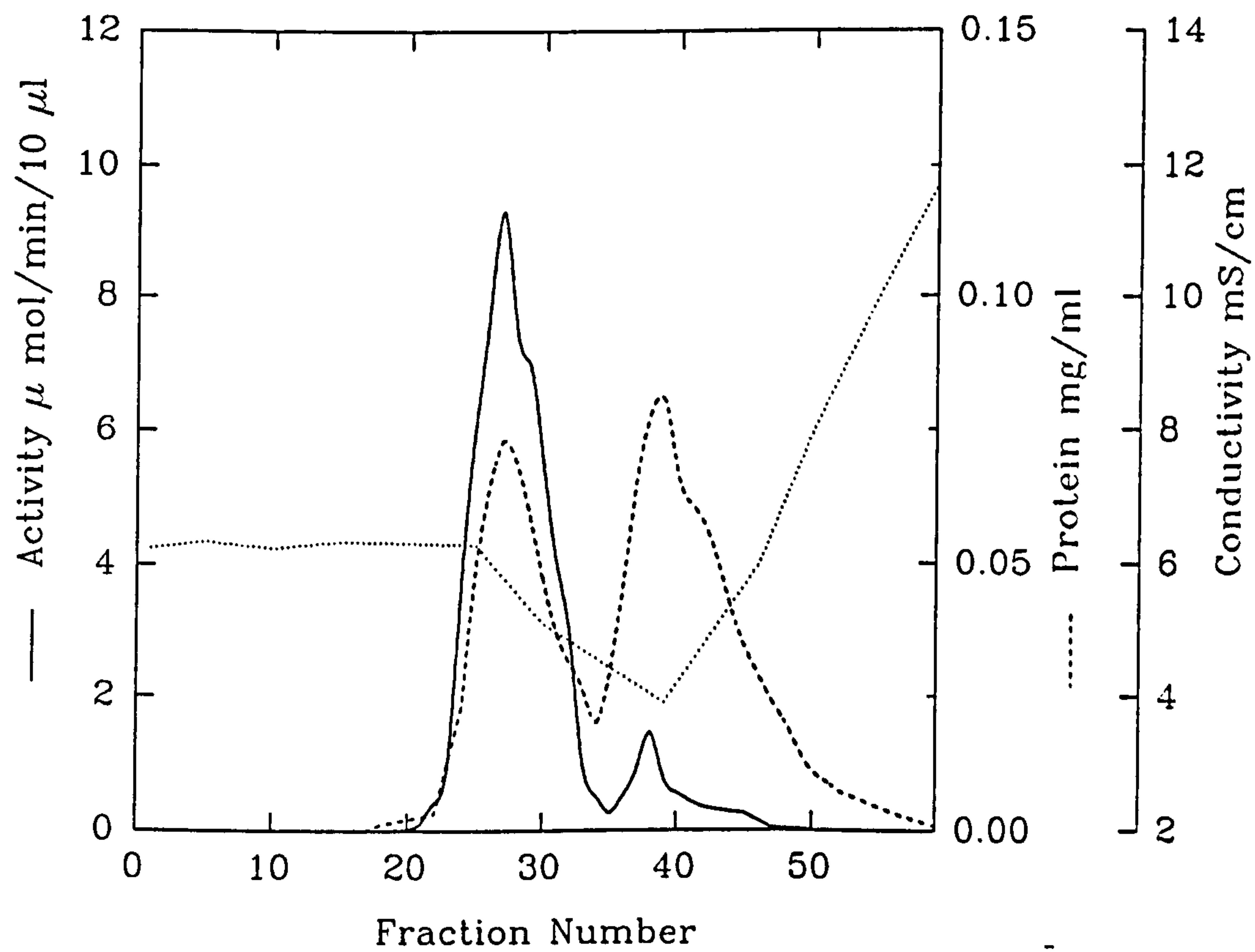


FIGURE 5.12

Elution profile of hydroxyapatite chromatography for esterase Est β 1 from the Trinidad strain.

1 ml fractions of the elution were collected. The sample containing the esterases was applied to the column in 10 mM phosphate buffer (pH 6.8) with 50 mM NaCl and eluted with a 10-200 mM phosphate buffer gradient without NaCl.



1980; Callaghan, 1989), and using the classification proposed by Karunaratne (1994) and adopted by Vaughan & Hemingway (1995), this esterase was named Est α 3. An Est α 3 was also found in the Habana strain (see Chapter 2) but this did not bind to the Q-Sepharose as did the Colombia and Trinidad Est α 3s, but eluted whilst washing with the homogenisation buffer (see section 2.3.4.). This suggests a marked difference in the surface charge of the Habana Est α 3 when compared to the Colombia and Trinidad Est α 3s. In purification trials with the MRES strain Est β 1 was also found in association with elevated Est α 3. It is interesting that esterases α (Est α 2, Est α 3 and non-elevated PelSS Est α 3) in the various *C. quinquefasciatus* strains all eluted from the phenyl-Sepharose as the first activity peak (see section 2.3.4., FIGURE 2.8; (Jayawardena, 1992; Karunaratne, 1994)), whilst the esterases β (Est β 1, Est β 2 and non-elevated PelSS Est β 1³) all eluted as the second activity peak. This suggests that hydrophobic interaction (with phenyl-Sepharose) can be added to immunological cross-reactivity and substrate preference (for α - or β -naphthyl acetate) as characteristics separating the esterases α from the esterases β and, therefore, provides more evidence towards the hypothesis that the two esterase types form distinct multi-allelic families (see section 4.3.2.). For the elution profile of Q-Sepharose chromatography for Trinidad Est α 3 and Est β 1 (FIGURE 5.10), two activity peaks are discernible. This was not an anomaly of a single Q-Sepharose chromatography run, the same pattern being seen in all three purifications from this strain. When aliquots of fractions lying within these peaks were run on a native PAGE gel stained for esterase activity, both proved to contain Est α 3 and Est β 1. It is possible that the two peaks represented different isoenzymes of these esterases with a slightly different surface charge. However, if this is the case then the

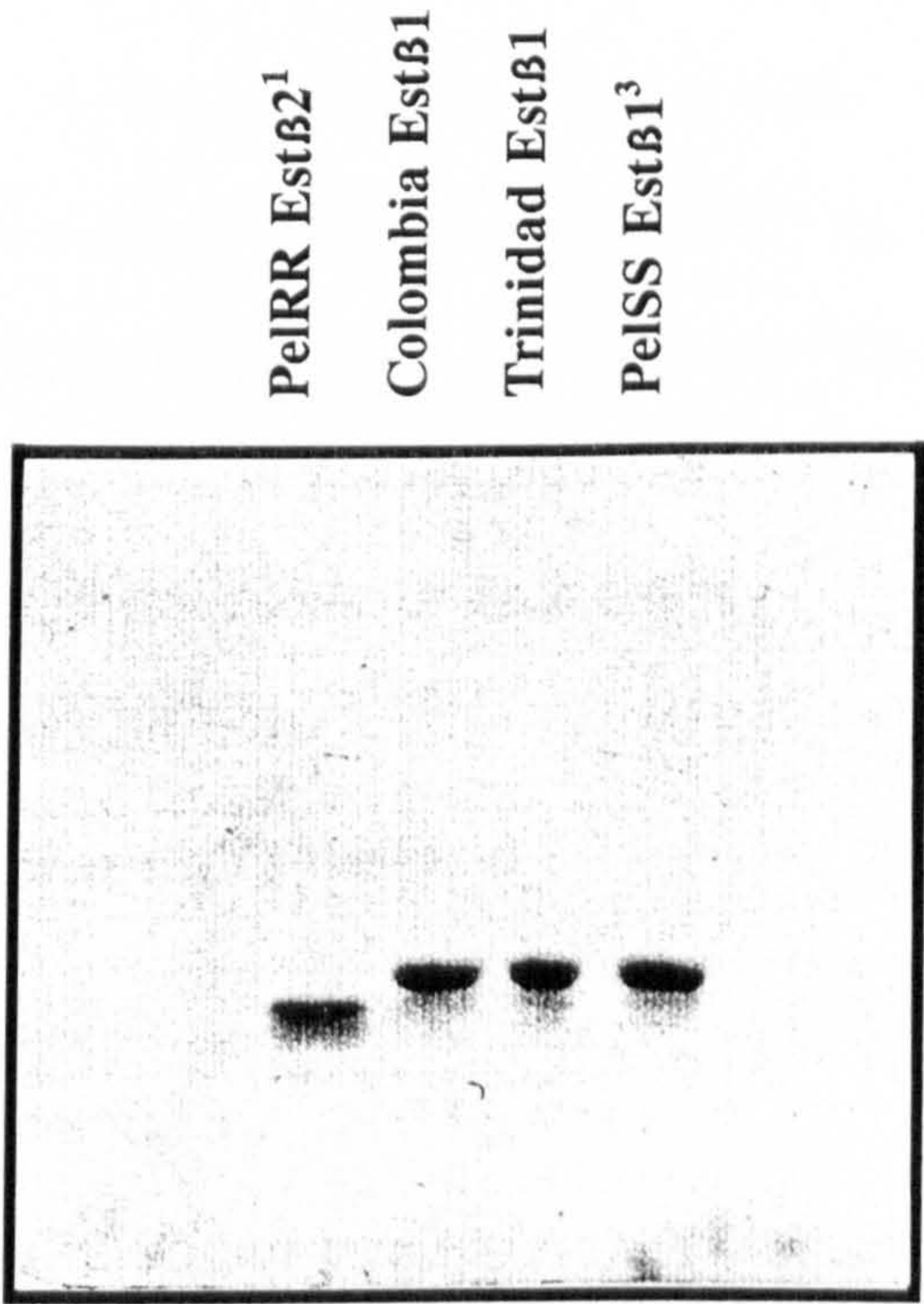
difference in overall charge must have been small as no separate Est α 3 or Est β 1 bands were discernible on native PAGE gels. The low yield of Est β 1 from the Trinidad strain meant that it was not feasible to pool the two peaks separately for the subsequent characterizations. Therefore, the two peaks were combined into a single pool for application to phenyl-Sepharose. For the profile of elution of activity from the hydroxyapatite in the purification of Trinidad Est β 1 (FIGURE 5.12), a large peak is followed by a second smaller peak. Again, aliquots of these peaks were run on native PAGE gels stained for esterases activity and both peaks proved to be Est β 1. It is possible that this separation into two peaks again represented the separation of different isoenzymes of Est β 1 which interacted slightly differently with the hydroxyapatite. The second peak was not included in the fractions pooled for further purification on *p*-chloromercuribenzoate-agarose as this peak coincided with a large peak of contaminant proteins. Data for single representative purification procedures for both the Colombia and Trinidad Est β 1s are presented in TABLE 5.2. The yield of Est β 1 from the Colombia strain was much greater than that for the Trinidad strain, reflecting the different degrees of amplification seen on southern-blots of *Eco*RI digested genomic DNA from the two strains (see section 5.3.3.). The specific activities of the Colombia and Trinidad Est β 1s are similar both to each other, and to that obtained for the Habana Est β 1 (see TABLE 2.2.). As with previous purifications, every preparation of Est β 1 from both strains was checked for purity on SDS/PAGE (results not shown). Aliquots of the concentrated enzyme were also run on native PAGE gels to check that the Est β 1s had been separated from all other esterases (FIGURE 5.13). The PelRR esterase Est β 2¹ and the PelSS esterase Est β 1³ were run on the same gel for comparison. It can be seen that the

TABLE 5.2. Purification of the esterase Estβ1 from the Colombia and Trinidad strains. The purification was monitored by the assay of 1 mM *p*-nitrophenyl acetate at 22°C except for the hydroxyapatite step for the Trinidad strain, and the *p*-chloromercuribenzoate step for both strains, in which esterase activity was monitored by the assay of 1 mM *p*-nitrophenyl caproate.

Step	Mosquito Strain	Specific Activity (Units/mg)	Protein (mg)	Purification (fold)	Recovery (%)
10,000 g supernatant	Colombia	0.26	760.73	-	-
	Trinidad	0.10	988.26	-	-
Q-Sepharose	Colombia	Estα3/Estβ1	96.25	6.28	79.47
	Trinidad	" / "	129.04	4.73	61.72
phenyl-Sepharose	Colombia	Estβ1	4.84	41.15	26.16
	Trinidad	"	6.57	19.72	13.12
Hydroxyapatite	Colombia	Estβ1	1.49	81.53	15.98
	Trinidad	"	0.61	135.14	8.40
<i>p</i> -chloromercuribenzoate-agarose	Colombia	Estβ1	0.24	251.23	7.90
	Trinidad	"	0.025	256.51	0.66

FIGURE 5.13

A native PAGE gel of Est β 1 purified from the Colombia (Lane 2) and Trinidad (Lane 3) strains with Est β 1³ purified from the susceptible PelSS strain (Lane 4) stained for esterase activity. Purified Est β 2¹ from the PelRR strain was run on the same gel for comparison in Lane 1.



Colombia and Trinidad Est β 1s have a relative mobility similar to that of the non-elevated PelSS Est β 1³.

5.3.5. INSECTICIDE INTERACTIONS OF COLOMBIA AND TRINIDAD Est β 1 WITH INSECTICIDES

The higher yield of purified Colombia Est β 1 enabled the deacylation rates (k_3 s) of this esterase to be determined with malaoxon, paraoxon and propoxur. After esterase activity of the reactivating enzyme was measured, the percentage activity was plotted against time and the slope of the regression line through data points used to obtain the reactivation constant, k_3 (FIGURE 5.14). Values for this constant are presented in TABLE 5.3 together with values for PelRR Est α 2¹ (Karunaratne, 1994), PelRR Est β 2¹ (Karunaratne *et al.*, 1993) and the aphid esterase E4 (Devonshire & Moores, 1982). The k_3 of the Colombia Est β 1 for paraoxon is significantly higher than those for either of the elevated PelRR esterases indicating a slightly higher rate of deacylation. However, taken together, the values of k_3 for the three elevated *C. quinquefasciatus* esterases are similar and confirm that the role of elevated Est β 1, as with elevated Est α 2 and Est β 2, is one of sequestration; the rapid binding of insecticide preventing it reaching the target site, AChE, followed by a slow insecticide hydrolysis step. For malaoxon, the rate of hydrolysis by the aphid esterase E4 is 50-fold higher than that of Colombia Est β 1, showing that, in addition to sequestration, hydrolysis is important in organophosphate detoxification by E4. This was emphasised in a comparison between the effectiveness of the elevated Est α 2¹/Est β 2¹-based mechanism of OP resistance in

FIGURE 5.14

Reactivation of the esterase Est β 1 purified from the Colombia strain after inhibition by paraoxon, malaoxon and propoxur. After the acylated enzyme was separated from unbound insecticide, its reactivation was measured and plotted against time. Linear regression was performed through data points and the reactivation constant, k_3 , obtained from the slope of each line.

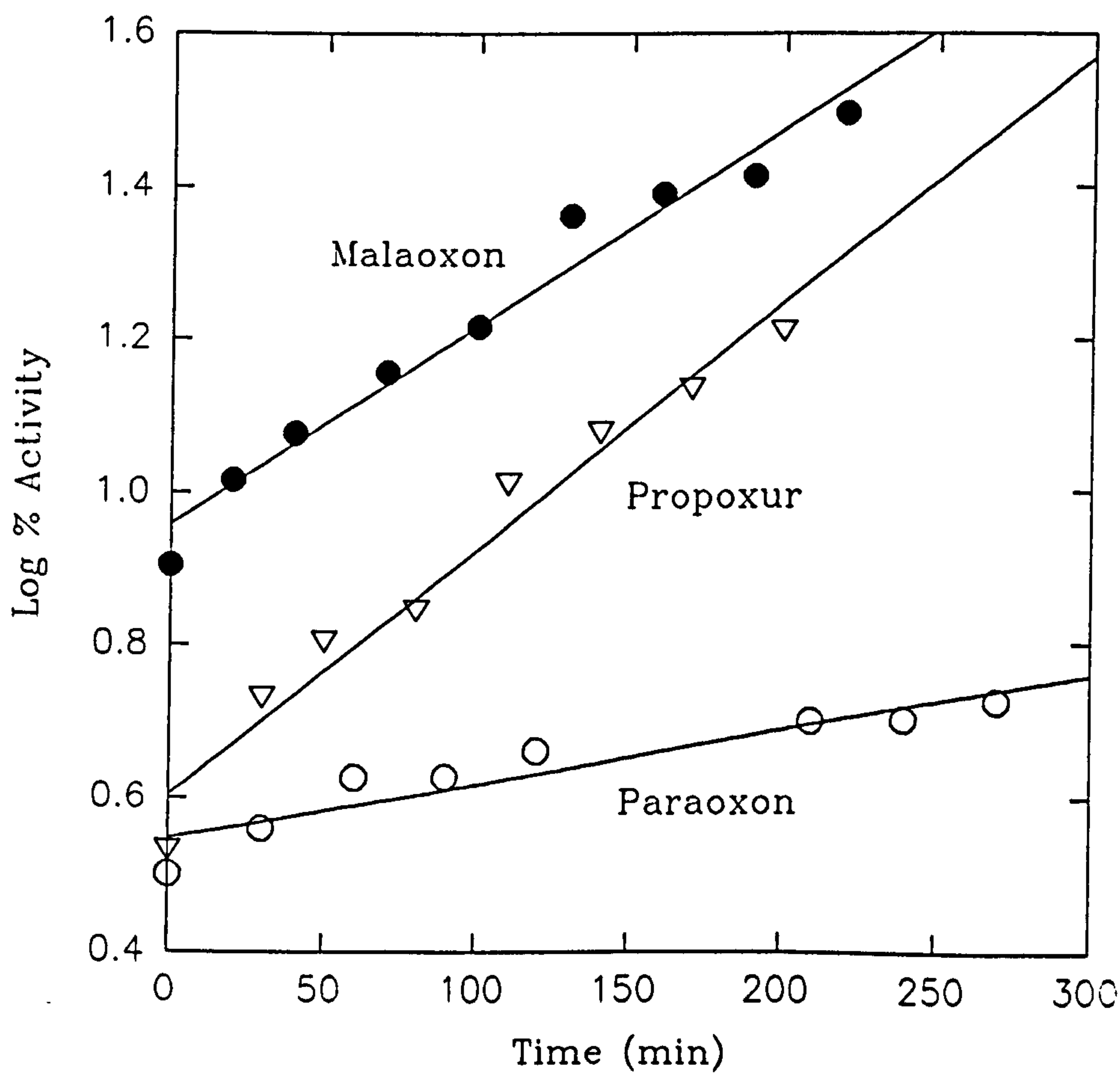


TABLE 5.3. Comparison of the deacylation rates (k_3) of Colombia Estβ1 with those of PelRR Estα2¹ (Karunaratne, 1994), PelRR Estβ2¹ (Karunaratne *et al.*, 1993) and aphid esterase E4 (Devonshire & Moores, 1982).

Insecticide	10 ⁴ x k_3 (min ⁻¹)			
	Colombia Estβ1	PelRR Estα2 ¹	PelRR Estβ2 ¹	Aphid E4
Malaoxon	10.1 ± 4.3	6.3	18.1 ± 3.7	520 ± 40
Paraoxon	25.5 ± 0.5	5.2	5.1 ± 2.0	55 ± 5.0
Propoxur	33.1 ± 1.3	62.3	10.9 ± 4.4	15.5 ± 2.0

Each mean value was calculated from three replicates, each replicate using

Estβ1 from a different purification.

the PelRR strain with the elevated E4 mechanism of resistance in the resistant G6 variant of the peach-potato aphid in sequestering and hydrolysing paraoxon (Karunaratne *et al.*, 1993). This showed that, whilst the amount of Est α 2¹ and Est β 2¹ in a PelRR larva (7.67 pmol) (Karunaratne *et al.*, 1993) and the amount of E4 in a G6 aphid (10 pmol) (Devonshire & Moores, 1982) are similar, and the amount of paraoxon that can be sequestered by them is also similar (2.1 ng and 2.5 ng respectively), the amount of the sequestered paraoxon that could be hydrolysed was 0.11 ng/h (5.59%) for elevated Est α 2¹/Est β 2¹ (Karunaratne *et al.*, 1993) and 0.83 ng/h (33.2%) for elevated E4 (Devonshire & Moores, 1982), almost a 6-fold difference. It has been hypothesised that herbivorous insects are pre-adapted to evolve resistance because their detoxifying enzymes must normally handle plant secondary chemistry (Mallet, 1989), including plant toxins produced to deter feeding. In line with this hypothesis, it was suggested that the elevated esterase-based mechanism in aphids may have evolved primarily as an adaptation to toxic compounds found in plants on which they feed and that this might explain its greater efficiency in dealing with organophosphates (Karunaratne *et al.*, 1993). Indeed, a susceptible variant was found to have an esterase that could hydrolyse 25% of sequestered paraoxon per hour, an efficiency similar to that of the esterase E4 (Devonshire & Moores, 1982), suggesting that the physiological role of this esterase maybe one of detoxification. One could extend this hypothesis (Karunaratne *et al.*, 1993) still further to explain why E4, which is kinetically identical to the equivalent esterase in a susceptible variant (Devonshire, 1977), is seemingly "pre-adapted" to hydrolyse permethrin and interacts reversibly with other pyrethroids, the structure of these pyrethroids being based on those of naturally

occurring pyrethrins found in the *Pyrethrum* genus of flowering plants. Whilst it is has been found that only the (1*S*) *trans* enantiomer of permethrin can be hydrolysed by E4, hydrolysis of the other isomers, and of deltamethrin (its α *R* diastereoisomer) being undetected, both deltamethrin and kadethrin bind reversibly with E4 (at both its catalytic site and other subsite(s)), and it was suggested that, in view of the very large amounts of E4 present in very resistant variants (3% of total protein (Devonshire & Moores, 1982)) that such binding, whilst being different to that with organophosphates and carbamates, could explain the strong general cross-resistance of some aphid variants to pyrethroids (Devonshire & Moores, 1989). It has also been hypothesised that mosquitoes, in which insecticide resistance is common, may also be pre-adapted through having evolved detoxification mechanisms to cope with organic chemicals leached from plant material in the stagnant water of their larval habitat (Mallet, 1989). However, there are several apparent shortcomings to these hypotheses: Firstly, as was pointed out by Callaghan *et al.* (1990), there are no data to suggest that the principal systems of detoxifying enzymes, among them being the esterases, are more active or more abundant in herbivorous than in predatory or parasitic insects; secondly, several species now resistant to a variety of insecticides (eg. the sheep blowfly *L. cuprina* (McKenzie & Whitten, 1982; Whyard *et al.*, 1994)) have barely any contact with plant materials (Devonshire & Moores, 1982); and finally, although an attempt has been made to investigate the role of elevated Est α 2¹ from the PelRR strain of *C. quinquefasciatus* with physiological substrates (Ketterman *et al.*, 1992), the role of this and other insect esterases involved with insecticide resistance has yet to be determined and there is, therefore, as yet, no hard evidence to suggest that the physiological role of these esterases is one of

detoxification.

The values of k_a for insecticide interactions with the Colombia and Trinidad Est β 1s are presented in TABLE 5.4. Values for Habana Est β 1 (Small *et al.*, 1995), non-elevated PelSS Est β 1³ (Karunaratne *et al.*, 1995a) and PelRR Est β 2¹ (Karunaratne *et al.*, 1993) are also presented for comparison. For both malaoxon and fenitrooxon Colombia Est β 1 had a higher rate of binding than Trinidad Est β 1. Indeed, for these two insecticides, significant differences were found between all three elevated Est β 1s. This result is further evidence supporting the hypothesis that esterase Est β 1 was amplified independently more than once. It also confirms that electrophoretic mobility of *C. quinquefasciatus* esterases is an extremely poor indicator of the different isoenzymes present in different strains, and reinforces the validity of the change in classification from that of Raymond *et al.* (1987) to that proposed by Karunaratne (1994) and adopted by Vaughan & Hemingway (1995). The reactivity of the non-elevated PelSS Est β 1³ is significantly different from those of the elevated Est β 1s for all the insecticides tested. As with the Habana Est β 1 (Small *et al.*, 1995), the Colombia and Trinidad Est β 1s have k_a s far higher for interactions with chlorpyrifos-oxon, paraoxon and propoxur than the non-elevated Est β 1³ purified from the susceptible PelSS strain (Karunaratne *et al.*, 1995a) and show, in terms of interactions with these insecticides, a greater similarity to the elevated Est β 2¹ (Karunaratne *et al.*, 1993) than they do to the non-elevated isozyme from the susceptible PelSS strain. These results are in agreement with the hypothesis that the elevated esterase-based mechanism confers resistance through amplification of alleles coding for esterases which have a greater specificity for the insecticides they

TABLE 5.4. The kinetic constant k_a for insecticide interactions with esterase Est β 1 purified from the Colombia and Trinidad strains.

Data for Habana Est β 1 (Small *et al.*, 1995), PelSS Est β 1³ (Karunaratne *et al.*, 1995a) and PelRR Est β 2¹ (Karunaratne *et al.*, 1993) are presented for comparison. The data are means \pm standard deviations. In the same row, different superscript letters indicate a significant difference ($P < 0.05$). Each mean was calculated from at least 10 k_a values.

Insecticide	$k_a \times 10^{-5} \text{ (M}^{-1} \text{ min}^{-1}\text{)}$				
	Colombia Est β 1	Trinidad Est β 1	Habana Est β 1	PelSS Est β 1 ³	PelRR Est β 2 ¹
Malaoxon [*]	1.30 ^a ± 0.13	0.07 ^b ± 0.01	0.16 ^c ± 0.02	0.40 ^d ± 0.05	0.50 ^d ± 0.17
Fenitrooxon [*]	7.09 ^a ± 1.28	0.22 ^b ± 0.03	1.52 ^c ± 0.32	0.33 ^d ± 0.01	1.60 ^c ± 0.30
Chlorpyrifos-oxon [*]	1230 ^a ± 203	1210 ^a ± 248	1340 ^a ± 88	2.29 ^b ± 0.81	1550 ^a ± 140
Paraoxon [*]	100.0 ^a ± 6.90	73.80 ^a ± 9.33	161 ^a ± 35.7	1.94 ^b ± 0.05	170.2 ^a ± 53.1
Propoxur [†]	0.0033 ^a ± 0.0005	0.0041 ^a ± 0.0007	0.0017 ^b ± 0.0002	<0.001 ^c	0.0052 ^a ± 0.0017
[*] Organophosphate [†] Carbamate					

The statistical test used to determine the significance of differences between mean values was that described in section 2.3.5.2.3.

sequester than the esterases coded for by their non-amplified counterparts (see section 2.4.). It is notable that, although the Trinidad and Habana strains are known to have been under selection pressure with malathion for many years in the field, the k_a s for the interaction of the Est β 1s from both strains with malaoxon are significantly lower than for the non-elevated PelSS Est β 1³. This observation would seem to go against the above mentioned hypothesis. However, there exists the possibility that the amplification the est β 1 genes occurred in other geographical locations where the *C. quinquefasciatus* populations were under selection pressure from other organophosphorus insecticides and that they have arrived at their present locations through migration of mosquitoes possessing them. The study of the est β 2 by Raymond *et al.* (1991) has already suggested that *C. quinquefasciatus* are capable of migrating very large distances. Although these esterases have a low efficiency for the binding of malaoxon, they can still confer insecticide resistance by virtue of their elevation. As with the Habana esterases Est α 2, Est β 1 and Est β 2 (see sections 2.3.5.2.3. and 3.3.2.2.3.), with elevated Est α 2s and Est β 2s purified from other strains, and with the non-elevated Est α 3 and Est β 1³ purified from the susceptible PelSS strain (Karunaratne *et al.*, 1993; Ketterman *et al.*, 1993; Hemingway *et al.*, 1993; Jayawardena *et al.*, 1994; Karunaratne *et al.*, 1995a), the higher reactivity of the Colombia and Trinidad Est β 1s with malaoxon over that with propoxur was reflected in a higher resistance ratio with malathion than with propoxur in insecticide bioassays (see TABLE 5.1).

5.4. SUMMARY

Combined bioassay and biochemical assay data suggest that the major mechanisms of OP resistance in the Colombia strain are elevated esterases and an altered AChE. In the Trinidad strain, only the elevated esterase-based mechanism of OP resistance was found. Whilst selection of elevated esterases in the Trinidad strain can be explained through the direct targeting of *C. quinquefasciatus* in Trinidad with both temephos and malathion as part of a vector control campaign, no such campaign has been waged against this mosquito in Colombia. Therefore, the selection of both the elevated esterase and altered AChE-based mechanisms in the Colombia strain has probably occurred via contamination of larval habitats by OPs used in the control of agricultural pests. On southern-blots of *EcoRI* digested Colombia and Trinidad genomic DNA hybridised with a ^{32}P -labelled *estB2*¹ cDNA probe, the restriction fragment pattern of the *estB1*s were found to be different both to each other, and to those of *estB1*s in the TEM-R (Raymond *et al.*, 1991) and MRES (Vaughan *et al.*, 1995) strains. A doublet of bands hybridising with the *estB2*¹ probe were detected in both the Colombia and Trinidad strains. Further experiments were suggested that would ascertain if the *estB1* differ from the TEM-R and MRES strains, and if the *EcoRI* site sits internally (in the coding sequence or introns) or in the flanking regions of the *estB1* gene. Evidence supporting the hypothesis that *estB1* has been amplified more than once (Vaughan *et al.*, 1995) came from the insecticide interactions of the EstB1s purified from the Colombia and Trinidad strains, these two esterases being readily distinguished on the basis of their k_a s. The greater intensity, on the southern-blots, of the Colombia *estB1* bands

showed that the degree of amplification of this gene in the Colombia strain is higher than in the Trinidad strain. This was also suggested by the higher yields of the purified enzyme from the Colombia strain when compared to the Trinidad strain. Variation in the *EcoRI* restriction fragment patterns of what are probably non-amplified *estB* genes between Colombia and Trinidad suggests a marked polymorphism in these genes. For both the Colombia and Trinidad strains, the elution profiles for esterases activity with phenyl-Sepharose showed the presence of a second activity peak which, on running aliquots of fractions lying within this peak on native PAGE gels stained for esterase activity, proved to be Est α 3. This esterase was not visible on native PAGE gels of crude homogenates, activity of this esterase being masked by that of the esterases Est β 1 which has a similar relative mobility. The deacylation rates (k_3 s) of the purified Colombia Est β 1 with malaoxon, paraoxon and propoxur were similar to values obtained for PelRR Est α 2¹ and Est β 2¹, and confirmed that the role of this esterase is one of sequestration. As with the Habana Est β 1 (see section 2.3.5.2.3.; Small *et al.*, 1995), the Colombia and Trinidad Est β 1s had k_a s far higher for interactions with chlorpyrifos-oxon, paraoxon and propoxur than the non-elevated PelSS Est β 1³ (Karunaratne *et al.*, 1995a) and, in terms of these insecticides, showed a greater similarity to the elevated Est β 2¹ (Karunaratne *et al.*, 1993). These results are in agreement with the hypothesis that the elevated esterase-based mechanism confers resistance through amplification of alleles coding for esterases having a higher reactivity with the insecticides they sequester than the esterases coded for by their non-amplified counterparts. It was noted that, although the Trinidad strain has been under selection pressure from OPs for many years in the field, the k_a for the Est β 1 purified from this strain with malaoxon was lower

than for the non-elevated PelSS Est β 1³, thus providing possible evidence against the above mentioned hypothesis. As an explanation for this apparent discrepancy it was suggested that the amplification of the est β 1 in the Trinidad strain might have occurred in another geographical location where the *C. quinquefasciatus* populations were under selection pressure from other OPs and that this amplified est β 1 has arrived in Trinidad through the subsequent migration of mosquitoes possessing it.

CHAPTER 6

GENERAL DISCUSSION

6.1. ESTERASE-BASED RESISTANCE MECHANISMS

The most commonly found mechanisms of OP and carbamate resistance in insects are based on qualitative and/or quantitative changes in esterases, and alterations in the target site AChE making it insensitive to inhibition. Elevation of non-specific carboxylesterases is a mechanism of OP and carbamate resistance in a variety of insect species. It has been hypothesised that phytophagous insects may be pre-adapted to evolve resistance because their detoxifying enzymes must normally handle plant secondary chemistry (Mallet, 1989) and section 5.3.4.). It could also be argued that mosquito larvae may be pre-adapted to evolve resistance because their detoxifying enzymes must handle the products leaching from decaying material including these same plants. However, as yet there is no definitive evidence to show that the major physiological role of the non-specific esterases is one of detoxification. Possible evidence, however, may come from a study currently being carried-out to localise the elevated Est α 2¹ and Est β 2¹ in larvae of the OP resistant PelRR strain, and non-elevated Est α 3 and Est β 1³ in the susceptible PelSS strain using antisera raised against the PelRR esterases. Preliminary data for the localisation of Est α 2¹ in PelRR larvae shows that the enzyme is concentrated in cells in the cuticle, salivary gland, Malpighian tubules, and possibly, the gut (J. Hemingway, personal communication). This localisation would ideally position the

elevated esterases (assuming Est β 2¹ is localised identically) to sequester toxins as they enter the larva. However, with respect to OPs, it raises the question as to whether the *P*⁴⁵⁰s would have activated the insecticides to their oxon form before encountering high concentrations of the esterases. Another possible role of the *Culex* esterases is fatty acid metabolism as both Est α 2¹ and Est β 2¹ can hydrolyse intermediate length mono- and diacylglycerols (Kettermann *et al.*, 1992; Jayawardena, 1992).

In the present study, characterisation of purified Est β 1 from the Habana, Colombia and Trinidad strains, and characterisation of Est α 2 and Est β 2 purified from the Habana strain, together with data for the characterisation of Est α 2 and Est β 2 purified from other strains, has revealed that the role of these elevated esterases is one of sequestration, the rapid binding of insecticides followed by slow turnover. This mechanism is similar to that for elevated E4 in *Myzus persicae* (Devonshire, 1977; Devonshire & Sawicki, 1979; Devonshire & Moores, 1982). It has been estimated that 0.4% of an OP resistant PelRR larva's total protein is made up of elevated Est α 2¹ and Est β 2¹ (Karunaratne *et al.*, 1993), and that 3% of an insecticide resistant aphid's total protein is of elevated E4 esterase (Devonshire & Moores, 1982). Therefore, a considerable amount of the insects protein synthesising machinery is given over to the production of these esterases. The advantage to insects possessing them when under insecticide selection pressure outweighs the disadvantage in terms of energy costs. However, the decline in the frequency of the elevated esterase-based mechanism in some field populations of *C. quinquefasciatus* suggests that its possession puts insects at a selective disadvantage when spraying of OPs ceases

(Rodriguez *et al.*, 1993). In *Myzus persicae*, a feedback mechanism has evolved that avoids the unnecessary cost of producing large amounts of E4 esterase when no OPs are being sprayed. When the E4 gene is being expressed, two *Msp*I restriction sites in the second exon of the gene are methylated (Field *et al.*, 1989; Field *et al.*, 1993). However, in some field populations (presumably not sprayed with OPs for some generations), these sites are not methylated causing a decrease in E4 mRNA levels and a loss of resistance. Reselection of these populations with insecticide caused a rapid return of both methylation and resistance (Field *et al.*, 1989). With *Myzus*, the increase in esterase expression once exposure to insecticide has resumed does not occur within a single generation, but over several generations. The insecticides can not, therefore, be said to be acting as classical inducers which produce a response within a generation. The existence of feedback mechanisms would imply that there must be a mechanism in the insects for detecting the presence of insecticides in their bodies. Possibly the insecticides present in the bodies of female aphids are detected by nymphs developing inside them, inducing them to overproduce the OP resistance related esterases. In the tsetse fly *Glossina morsitans morsitans*, the larva developing within the adult female fly constitutes a major sink for the juvenile hormone mimic pyriproxyfen following movement through the fat body and the uterine gland (Langley *et al.*, 1990). If the nymphs developing within an aphid act as sinks for OPs, they would actually be under a greater selection pressure than their parent. Indeed, with several generations being present in a parthenogenetic female aphid at one time, the insecticide picked up by an adult could be concentrated several times over in the nymphs developing within it. But why is there a delay in the 'switching on' of overproduction of esterases when, as a survival

mechanism, it would be more efficacious to be able to produce the esterases whenever the insect comes into contact with insecticides? Maybe the mechanism simply is not that efficient. It is certainly an interesting question and one worthy of further investigation.

On the basis of the identical restriction fragment patterns of the amplified *est*β2 gene and its flanking regions from African, Asian and North American populations of *C. quinquefasciatus* it has been hypothesised that the *est*β2 gene has been amplified only once and has subsequently spread worldwide by migration (Raymond *et al.*, 1991). As mentioned in section 3.1., if this is so, one would expect the expressed Estβ2 in all *C. quinquefasciatus* strains having this amplified esterase gene to be identical and, therefore, their biochemical characteristics also to be identical. Since Estα2 has always been found co-amplified with Estβ2 (Raymond *et al.*, 1991) and since the *est*α2¹ gene is situated on the same amplicon as the *est*β2¹ gene (Vaughan, 1995), the same should be true of this esterase. As Estα2 and Estβ2 should be identical, the strains carrying them (and no other OP resistance mechanisms) should also have similar cross-resistance spectra to organophosphorus insecticides. However, *k_as* for insecticides have been observed to vary with elevated Estα2 and Estβ2 purified from strains of different geographic origin (Karunaratne *et al.*, 1993; Kettermann *et al.*, 1993; Jayawardena *et al.*, 1994; the present study), and insecticide cross-resistance spectra of *C. quinquefasciatus* strains from different geographical areas which have only the elevated esterase Estα2/Estβ2-based mechanism of organophosphorus insecticide resistance have also been seen to vary (Georghiou & Pasteur, 1978; Amin & Peiris, 1990; Hemingway *et al.*, 1990; Peiris

& Hemingway, 1990a; Peiris & Hemingway, 1990b). Southern blots of PelRR and PelSS *EcoR*I genomic digests probed with cDNA fragments of the PelRR *est* α 2¹ and *est* β 2¹ esterase genes (Karunaratne *et al.*, 1995a) showed that the restriction fragment of the amplified *est* β 2¹ gene in PelRR was the same size as that reported by Raymond *et al.* for *est* β 2 in other strains (Raymond *et al.*, 1991) but also, that the PelRR strain contains non-amplified *est* α and *est* β genes. It is unlikely that the purification methodologies employed would have separated the expressed esterases of these genes from the elevated esterases. It was, therefore, suggested that variation in the observed k_a s of the enzyme preparations from strains of different geographic origin having elevated Est α 2 and Est β 2 might be due to minor contamination with different non-elevated esterases (Karunaratne *et al.*, 1995a), and/or to different levels of amplification of *est* α 2 and *est* β 2 with concomitant variation in the proportion of non-elevated esterases present. Indeed, whilst only a single non-amplified *est* α and *est* β allele was detected in the PelSS strain (Karunaratne *et al.*, 1995a), restriction fragment analysis of non-amplified *est* α and *est* β alleles in field populations have shown a high level of polymorphism (Raymond *et al.*, 1991). Since the PelRR strain still contains non-amplified *est* α and *est* β alleles, it is possible that other non-amplified alleles might exist in other populations of *C. quinquefasciatus* having co-amplified *est* α 2 and *est* β 2. This might also explain the observed variation in cross-resistance spectra.

In contrast to the restriction fragment patterns for the amplified *est* β 2 genes in *C. quinquefasciatus* strains of different geographic origin, which were identical (Raymond *et al.*, 1991; Karunaratne *et al.*, 1995a), the restriction fragment patterns

for two amplified *estB1* genes, *estB1*¹ in the Californian TEM-R strain (Mouches *et al.*, 1990) and *estB1*² in the Cuban MRES strain differed (Vaughan *et al.*, 1995). Moreover, the nucleotide and amino acid sequences of the two *estB1* cDNAs also differed (Vaughan *et al.*, 1995). On the basis of these data it was hypothesised that *estB1* has been amplified independently more than once (Vaughan *et al.*, 1995). Significant differences were found between the k_a s of the elevated EstB1s from the Habana, Colombia and Trinidad strains for both malaoxon and fenitrooxon. In addition, the *EcoRI* fragment sizes of the Colombia and Trinidad *estB1*s and flanking regions were different to those amplified in the TEM-R (Raymond *et al.*, 1991) and MRES (Vaughan *et al.*, 1995) *estB1*s. These results suggest that esterase *estB1* has been amplified independently at least three (and possibly four or five if Colombia and Trinidad *estB1* restriction fragment lengths are shown to differ) times.

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Non-elevated A- and B-type esterases (1953a; 1953b; 1993) can bring about resistance to OPs and pyrethroids by virtue of their rapid hydrolysis of insecticide molecules into less toxic and more readily excretable forms. For example, in both humans and rabbits, resistance to paraoxon is due to the presence of serum paraoxonases which fall into the A-type category (Du *et al.*, 1993; Furlong *et al.*, 1993; Li *et al.*, 1993). In several examples of resistance based on non-elevated esterases the enzymes have been found to be pre-adapted to the metabolism of certain insecticides. Both the porina moth *Wiseana cervinata* and the whitefly *Bemisia tabaci* have esterases which give tolerance to pyrethroids in field populations that are naive to these insecticides (Chang & Jordan, 1983; Ishaaya *et al.*, 1987). In the small brown planthopper *Laodelphax striatellus*, fenitrothion

resistance is due to a quantitative change in a carboxylesterase that is capable of hydrolysing the insecticide, this carboxylesterase being kinetically identical to that in a susceptible strain (Sakata & Miyata, 1994). This pre-adaptation of the esterase to hydrolyse fenitrothion might again be cited as evidence supporting the hypothesis that the physiological role of these esterases is one of detoxification (Mallet, 1989).

It was suggested in section 2.4. that elevated esterase-based OP resistance in mosquitoes of the *C. pipiens* complex may be due to amplification of alleles previously present in populations that coded for proteins which were pre-adapted to have a higher affinity for OPs. After *Hind*III digestion of genomic DNA of single mosquitoes, a high level of polymorphism was detected in *est*β alleles in susceptible populations from France and Portugal (Raymond *et al.*, 1991). However, neither the unamplified *est*β1 nor the unamplified *est*β2 were detected. There are two possible explanations for this: Firstly, given the high level of polymorphism of the unamplified *est*β alleles [at least 15 alleles in a sample of 30 insects from a sample of individuals from a population in France and 13 (all different from the 15 detected in the French population) in a sample of 28 mosquitoes from a population in Portugal], it is possible that the unamplified *est*β1 and *est*β2 alleles were present in both populations but, due to chance, these were missed in the samples of mosquitoes taken; and secondly, if the hypothesis regarding the migration of the co-amplified *est*β2 and *est*α2 is correct, it is possible that these were amplified at another geographic location. It is notable that only a single non-amplified *est*β alleles and two non-amplified *est*α alleles (*est*β1³ in both PelSS and PelRR, *est*α3 in PelSS and an *est*α in PelRR) were detected in mosquitoes derived from a single

Sri Lankan population (Pel) (Vaughan *et al.*, 1995; Vaughan & Hemingway, 1995; Karunaratne *et al.*, 1995a). This is in contrast to the levels of polymorphism one would have expected on the basis of the findings of Raymond *et al.* (1991). It is possible that high levels of polymorphism do not exist in the non-amplified alleles of *est* α and *est* β in Sri Lankan populations of *C. quinquefasciatus*. However, an alternative explanation is that such polymorphism exists but that, during sampling or colonisation, it was somehow lost. It is also possible that other non-amplified *est* α and *est* β alleles exist in both the PelSS and PelRR populations but that they are present at only a low frequency. Non-amplified esterases did not produce a high enough signal to be detected on Southern blots of genomic DNA from an individual insect and detection of these alleles had to be carried out using genomic DNA extracted from several insects. Any particular non-amplified allele would, therefore, need to be present in several individuals before it would be detected. Given this, either of the possible reasons advanced to explain the non-detection of the non-amplified *est* β 1 and *est* β 2 alleles in the study of Raymond *et al.* (1991) could equally be advanced to explain the non-detection of the non-amplified *est* α 2¹ and *est* β 2¹ alleles in the studies of the molecular biology of Sri Lankan *C. quinquefasciatus* esterases (Vaughan *et al.*, 1995; Vaughan & Hemingway, 1995; Karunaratne *et al.*, 1995a).

6.2. COMPARISON OF THE MOLECULAR BIOLOGY OF *C. QUINQUEFASCIATUS* ESTERASES WITH THAT OF OTHER SERINE HYDROLASES

It has yet to be determined what amino acid differences between the 'susceptible' PelSS Est α 3 and Est β 1³ and the 'resistant' PelRR Est α 2¹ and Est β 2¹ and the other resistant esterases contribute to the observed difference in the affinity of these esterases for OP and carbamate insecticides. The PelRR Est α 2¹ has recently been expressed using the baculovirus system (Vaughan, 1995). This system is being increasingly used for expression, not least because it is highly versatile and gives a high level of expression of both prokaryotic and eukaryotic genes (Miller, 1988; Maeda, 1989). As the system is not only eukaryotic, but makes use of insect cell lines, it is likely to express the *Culex* esterases exactly as they are *in vivo*. It is hoped that, once the susceptible PelSS est α 3 has been sequenced, this same system can be used to express both the susceptible and resistant esterases α and β so that they can be used in site-directed mutagenesis studies to deduce those amino acid changes that are associated with varying insecticide binding affinity. The same system has been used successfully to express the juvenile hormone esterase of *Heliothis virescens* (Hammock *et al.*, 1990; Bonning *et al.*, 1994). It is also hoped to clone and sequence the esterases involved in OP resistance in the brown planthopper *Nilaparvata lugens* (Hemingway *et al.*, in press; Karunaratne and Hemingway, submitted), the malathion carboxylesterase in resistant *An. stephensi* (Hemingway, 1982) and their equivalents from susceptible populations of the same insect species and carry out similar site-directed mutagenesis studies on these. Before this,

however, more needs to be known about the three-dimensional structure of these esterases and how this relates to those already determined for *Torpedo californica* AChE (Sussman *et al.*, 1991) and *Geotrichum candidum* lipase (Schrag & Cygler, 1993) so that it can be decided which residues should be mutated. The baculovirus expression system would enable sufficient *Culex* esterases to be produced for purification, crystallization and determination of their three-dimensional structure. This would lead to a greater understanding of the action of carboxylesterase on insecticides and, it is hoped, increase our understanding of esterase structure and aid in the rational design of new insecticides.

Using the three-dimensional structures of *Torpedo californica* AChE (Sussman *et al.*, 1991) and *Geotrichum candidum* lipase (Schrag & Cygler, 1993), the amino acid sequences of these and 29 other α/β hydrolase fold proteins were aligned (Cygler *et al.*, 1993). This alignment showed that 24 residues were absolutely conserved in all the enzymes including the *C. quinquefasciatus* *estB1*¹ esterase (Mouches *et al.*, 1990) and an additional 49 are well conserved. The conserved residues include the active site, disulphide bridges, salt bridges and residues in the core of the proteins. However, in *C. quinquefasciatus* *estB1*¹ esterase (Mouches *et al.*, 1990) a cysteine residue at position 84 (Cys¹⁰⁵ and Cys⁹⁴ in *T. californica* and *G. candidum*, respectively), forming a disulphide bridge in *T. californica* AChE and *G. candidum* lipase (Cys⁶¹ and Cys⁶⁷), is not conserved, the residue at this position being a serine. There is also a serine at position 84 in *est* α 2¹, *estB1*² and *estB1*³ (Vaughan *et al.*, 1995; Vaughan & Hemingway, 1995). The Cys⁶⁵, which forms a disulphide bridge with Cys¹⁰⁵ and Cys⁹⁴ in *T. californica* and *G. candidum* respectively, is missing in

estα2¹ (Vaughan & Hemingway, 1995). It was suggested by Cygler *et al.* (1993) that the disulphide bridges in the serine hydrolases, by influencing the shape of these enzymes, play a role in substrate binding and/or substrate recognition. It is notable that the OP resistance related *Myzus persicae* esterase E4 has this disulphide bridge, as do the *Drosophila melanogaster* esterase 6 and P (Oakeshott *et al.*, 1987; Collet *et al.*, 1990) and the *Heliothis virescens* juvenile hormone esterase (JHE) (Hanzlik *et al.*, 1989). Therefore, the *C. quinquefasciatus* esterases appear to have lost this bridge which is conserved in other esterases. It is also notable that both the *estα2¹* and *estβ2¹* have nine cysteine residues, the majority of which are not conserved in other serine hydrolases (Vaughan *et al.*, 1995; Vaughan & Hemingway, 1995). The reason for this large number of cysteines is not known, but it has been suggested that five of these may not be involved with disulphide bridges (Jayawardena & Hemingway, 1995). This suggestion was prompted by the observation that purified *Estα2¹* and *Estβ2¹* stored without high concentrations of the reducing agent dithiothreitol (DTT) produced multiple anodal satellite bands on native PAGE gels. Inclusion of DTT prevented these bands from forming. Jayawardena and Hemingway (1995) suggested that the cysteines not involved in disulphide bridges can be oxidised resulting in multiple electrophoretic bands. In addition the amino acid sequence of *estα2¹* differs from other serine hydrolases at another highly conserved residue at position 37. In all the sequences aligned by Cygler *et al.* (1993) and in those of the esterases β (Vaughan *et al.*, 1995), the amino acid residue at this position is alanine, but in *estα2¹* this residue is a valine (Vaughan & Hemingway, 1995). The function of the alanine at this position and, therefore, the reason that it is so highly conserved, is unknown. The substitution of alanine for valine is not

considered to be conservative (Cygler *et al.*, 1993), but its effect on the structure of the enzymes is unlikely to be large, both alanine and valine having similar saturated hydrophobic side-chains.

An alignment of 24 amino acid residues around the active site serine of the *Culex* esterases and other serine esterases is shown in FIGURE 6.1. Considerable similarity is seen between the *Culex* esterases and other serine esterases. Eight of the residues are absolutely conserved in the sequences cited and, indeed, in all known sequences of serine hydrolases. A further eleven residues were conserved in the majority of cases. Ala¹⁹ is conserved in all but the mouse carboxylesterase (Ovnic *et al.*, 1991), Thr¹³ in all but the *Drosophila* esterases (Oakeshott *et al.*, 1987; Collet *et al.*, 1990), Ser²³ in all but the *Heliothis* JHE (Hanzlik *et al.*, 1989), and Val²⁴ in all but *Drosophila* Est P (Collet *et al.*, 1990) and *Heliothis* JHE (Hanzlik *et al.*, 1989). The active site Ser¹⁸ is part of a conserved sequence: Gly-Glu(His)-Ser-Ala-Gly-Ala/Gly (Cygler *et al.*, 1993). This sequence, and more particularly the sequence Gly-Xaa-Ser-Xaa-Gly (where Xaa can be one of several amino acid residues), has been found in many other enzymes that have a catalytic triad (Brenner, 1988). The active site serine is embedded in a tight turn between a β -strand and an α -helix and is in a strained conformation (Ollis *et al.*, 1992). As a result, the serine hydroxyl is presented in a position easily accessible to the catalytic histidine and also to the substrate. The requirement for glycine two residues before and after the serine is due to the close proximity of these residues in the tight turn from the strand to the helix. In *Torpedo* AChE, Asn¹ is just before a highly irregular helical turn formed by Ile² and Phe⁵ (Cygler *et al.*, 1993) but its specific function at

FIGURE 6.1. Identities between amino acid sequences surrounding the active site serine residue of the *Culex* esterases α and β and other serine esterases. Amino acids common to all 16 sequences are shown in bold type with the active site serine underlined. If an amino acid makes up the majority at a particular position it is shown in upper case.

Serine esterase	Sequence	Ref.
<i>Culex estB1</i> ¹	NIAAFGGDPkrVTLa Gh <u>S</u> AGaASV	1
<i>Culex estB1</i> ²	NIgAFGGDPkrVTLv Gh <u>S</u> AGaASV	2
<i>Culex estB1</i> ³	NIAAFGGDPkrVTLv Gh <u>S</u> AGaASV	2
<i>Culex estB2</i> ¹	NIAAFGGDPkrVTLv Gh <u>S</u> AGaASV	2
<i>Culex estα2</i> ¹	NvAnFGGDPkniTLF Gh <u>S</u> AGGcSV	3
<i>Myzus</i> E4/FE4	NIvAFGGDPkrVTit Gm <u>S</u> AGasSV	4
<i>Drosophila</i> Est 6	NIA s FGGePqnVlLv Gh <u>S</u> AGGASV	5
<i>Drosophila</i> Est P	NIA h FGGmPdnivLi Gh <u>S</u> AGGASa	6
<i>Heliothis</i> Juvenile Hormone Esterase	NaknFGGDPsdiTia Gq <u>S</u> AGasaa	7
Rat carboxylesterase	NIA n FGGnPdsVTiF Ge <u>S</u> AGGvSV	8, 9
Mouse carboxylesterase	NIA n FGGnPdsVTiF Ge <u>S</u> sGGiSV	10
Rabbit carboxylesterase	NIA h FGGnPksVTLF Ge <u>S</u> AGGtSV	11
<i>Anopheles</i> AChE	NakAFGGDPdliTLF Ge <u>S</u> AGGsSV	12
<i>Drosophila</i> AChE	NahAFGGnPewmTLF Ge <u>S</u> AGssSV	13
<i>Torpedo</i> AChE	NIqfFGGDPktVTiF Ge <u>S</u> AGGASV	14
Human AChE	NvAAFGGDptsVTLF Ge <u>S</u> AGaASV	15

1. Mouches *et al.*, 1990; 2. Vaughan *et al.*, 1995; 3. Vaughan & Hemingway, 1995; 4. Field *et al.*, 1993; 5. Oakeshott *et al.*, 1987; 6. Collet *et al.*, 1990; 7. Hanzlik *et al.*, 1989; 8. Long *et al.*, 1988; 9. Takagi *et al.*, 1988; 10. Ovnicek *et al.*, 1991; 11. Ozols, 1989; 12. Hall & Malcolm, 1991; 13. Hall & Spierer, 1986; 14. Schumacher *et al.*, 1986; 15. Soreq *et al.*, 1990.

this position and, therefore, the reason for its preservation, are not known. Phe⁵ in *Torpedo* AChE is at the end of helix $\alpha_{4,5}^2$ on a tight turn into strand β_5 which contains the active site serine at its end, and packs tightly against the proteins interior. Gly⁶ and Gly⁷ are at the start of the turn into strand β_5 and assume an unusual conformation packing closely against Ile² (Cygler *et al.*, 1993). Asp⁸ is hydrophilic and is exposed, whereas Pro⁹ is hydrophobic and faces the proteins interior. All these residues are conserved in the *Culex* esterases and, therefore, are likely to perform the same functions. The MRES *estB1*² differs from the other *Culex* esterases β in having a Gly at position 3 instead of an alanine. This substitution is, however, a conservative one and is unlikely to have any great effect on the properties of the protein. A more significant substitution is that seen at position 15 in the TEM-R *estB1*¹, in which an alanine is substituted for a valine. The TEM-R EstB1¹ has yet to be purified and characterised biochemically and it is not, therefore, known what effect this substitution has on the activity of the enzyme relative to the other EstBs.

In a comparison of the deduced amino acid sequence similarities of PelRR *estB2*¹ and several vertebrate esterases to PelRR *estA2*¹, and the immunological cross-reactivity of these esterases with the PelRR EstA2¹ antiserum (Jayawardena, 1992), Karunaratne (1994) observed that there was no direct relationship between the two. For example, the amino acid sequence similarities of PelRR *estB2*¹ (Vaughan *et al.*, 1995), human AChE (Soreq *et al.*, 1990) and rabbit liver esterase (Ozols, 1989) to PelRR *estA2*¹ were 70, 40 and 20% respectively, whilst the immunological cross-reactivities of these esterases with the PelRR EstA2¹ antiserum were 2, 0.1 and

>10% respectively (Karunaratne *et al.*, 1995b). It seems, therefore, that immunological cross-reactivity has less to do with overall amino acid sequence homology, than with the possession of specific epitopes on the surface of the esterase molecules which the antibodies in the serum can recognise. It is notable, however, that both the Habana Est β 1 and Est β 2 cross-reacted with the Est α 2¹ antiserum to a similar degree (see section 4.3.2.). Whilst the cDNAs for the Habana esterases β have not been sequenced, and the amino acid sequences are, therefore, not available, the amino acid sequence similarities of all *Culex* esterases β have been above 95%. Assuming this is also true of the Habana esterases β , the result suggests that the high degree of similarity at the amino acid level of these enzymes is reflected in their similar cross-reactivities with the Est α 2¹ antiserum.

6.3. THE AMPLIFICATION AND MIGRATION OF *CULEX* ESTERASE GENES

The *Culex* esterases Est α 2¹ and Est β 2¹ have always been reported as being co-elevated wherever they have been detected (Georghiou & Pasteur, 1978; Villani *et al.*, 1983; Hemingway *et al.*, 1990; Raymond *et al.*, 1991; Peiris & Hemingway, 1993; Rivet *et al.*, 1993). An explanation for this came with the recent discovery that the *est* α 2¹ and *est* β 2¹ genes are located on the same amplicon, in a head to head orientation, ie. they are inextricably co-amplified (Vaughan, 1995). Esterase Est β 1 has also been found in association with an amplified esterase, in this case Est α 3. Three strains, Colombia, Trinidad and MRES have all been found to have these two esterases co-elevated (see section 5.3.3.). However, as was seen in the immunoblot of individuals of the Habana strain possessing elevated Est β 1 (see

section 4.3.1.), this was not associated with a co-elevated Est α 3, although Est α 3 was found elevated by itself in this strain. In the TEM-R strain also, no elevated Est α was reported in association with elevated Est β 1¹ in immunoblot studies (Mouches *et al.*, 1987). These data suggest, therefore, that *est* α 3 and *est* β 1 are not on the same amplicon as this would explain why in some strains of *C. quinquefasciatus*, *est* α 3 and *est* β 1 are co-amplified and in others not. However, this has still to be confirmed by a molecular study of the chromosomal arrangement of these esterases genes.

The co-amplified *est* α 2 and *est* β 2 occur in populations of *C. quinquefasciatus* on at least three continents (Raymond *et al.*, 1991). The occurrence of esterase Est β 1 is more restricted being found in populations from California, Cuba, Central America and Asia (Pasteur *et al.*, 1981a; Bisset *et al.*, 1990; Georghiou, 1992). As co-amplification of *est* α 2 and *est* β 2 is more common than amplification of *est* β 1, it was thought possible that the esterases coded for by these genes have a higher affinity for organophosphates than Est β 1 and hence confer a greater selective advantage in the presence of these insecticides. Indeed, the spread of *est* α 2 and *est* β 2 into populations in California (Raymond *et al.*, 1987) and into those in and around Havana seemed to strengthen this hypothesis. However, as was reported in Chapter 2, this hypothesis proved incorrect, the Habana Est β 1 having a higher affinity for some insecticides than the PelRR Est α 2¹ and affinities closely similar to Est β 2¹. Another hypothesis that might be put forward is that elevation of two esterases, the Est α 2 and Est β 2, with slightly different insecticide kinetics, might be better than one, the Est β 1. However, as was mentioned above, three populations that possess amplified *est* β 1 have now been shown to possess amplified *est* α 3. Therefore, it seems

that this hypothesis also is incorrect. A third hypothesis was prompted by the recent discovery of a partial xanthine dehydrogenase (*XDH*) gene sequence on the same amplicon as *est*β2¹ and *est*β2¹ (Vaughan, 1995). *XDH* is a ubiquitous molybdenum hydroxylase which has a primary role in pteridine metabolism in insects (Hille & Nishino, 1995). *XDH*-null mutants of *Drosophila* are hypersensitive to the herbicide paraquat which generates free oxygen radicals, suggesting that this enzyme plays an important role in the oxygen defence system in insects (Hilliker *et al.*, 1992; Humphreys *et al.*, 1993). It has been hypothesised that the *XDH* gene, situated on the same amplicon as the *est*α2¹ and *est*β2¹ genes, contributes to the selective advantage that this amplicon confers (Vaughan, 1995). Given the polluted environment in which *Culex* larvae live, and the possible contamination of this environment with herbicides, amplification of a gene improving the mosquitoes oxygen free radical scavenging system could confer a major selective advantage which might pre-date that conferred by the elevated esterase-based OP resistance mechanism. This explanation may not only explain the displacement of elevated *Est*β1¹ in California (Raymond *et al.*, 1987) and *Est*α3/*Est*β1² in Cuba, but may also explain its displacement of elevated *Est*α1 in populations of *C. pipiens* in France and Italy (Bonning & Hemingway, 1991; Callaghan *et al.*, 1991; Callaghan *et al.*, 1993; Rivet *et al.*, 1993) (see section 7.4.) and co-elevated *Est*α4 and *Est*β3 in France (Poirie *et al.*, 1992). In mosquito populations in Lucca, Italy, co-elevated *Est*α2 and *Est*β2 were detected in 1985 (Callaghan, 1989) and by 1989 had completely displaced *Est*α1 (J. Hemingway, personal communication).

As mentioned in section 1.5.6., mosquitoes of the *Culex pipiens* complex contain full-

length and truncated copies of Juan-like elements (Mouches *et al.*, 1990; Agarwal *et al.*, 1993), belonging to the LINE superfamily of transposable elements. In particular, two truncated Juan elements are situated within the *estB1*¹ amplicon (Mouches *et al.*, 1990), and it has been hypothesised that this may be linked to the amplification process (Curtis, 1994). As yet, however, there is no direct evidence to support this hypothesis. To gain data on the mechanism(s) promoting amplification, it would be beneficial to sequence other *Culex est* gene amplicons, and also those of the *M. persicae* E4 and FE4 esterase genes, to look for common sequences that may be involved with this process.

6.4. THE TRANSFER OF AMPLIFIED ESTERASES BETWEEN 'SPECIES'

There is much debate as to whether *C. pipiens* and *C. quinquefasciatus* are two separate species or are parapatric sub-species. In the process of assigning five taxa, below species status, to the *C. pipiens* complex, *C. pipiens pipiens* and *C. quinquefasciatus* were resurrected as species (Knight, 1978) and this assertion was supported by observed variations in several morphometric indices (Ishii, 1980; Ishii, 1986; Jakob & Francy, 1984). Many taxonomists now hold that these are two separate species (Sirivanakarn & White, 1978), whilst others still refer to them as being part of a species complex (Dahl, 1988). Of the regions where the two species meet, hybridisation has been observed in the southern USA/Mexico, Saudi Arabia and China/Japan (Barr, 1965; Tabachnick & Powell, 1983), whilst in other regions, for example in South Africa, there is no evidence of hybridisation (Donaldson, 1979). Possible evidence of hybridisation between the two species is the appearance

of the co-elevated Est α 2 and Est β 2 in populations of *C. pipiens* in France and Italy (Bonning & Hemingway, 1991; Callaghan *et al.*, 1991; Callaghan *et al.*, 1993; Rivet *et al.*, 1993). In both countries, OP treatments had previously selected for elevated Est α 1 (Pasteur *et al.*, 1981a; Pasteur *et al.*, 1981b; Villani & Hemingway, 1987) and also co-elevated Est α 4 and Est β 3 in France (Poirie *et al.*, 1992). Co-elevated Est α 2 and Est β 2 were first detected in France in 1986 (Rivet *et al.*, 1993) and in Italy in a population from Lucca, Italy, collected in 1985 (Callaghan, 1989). It has yet to be confirmed by restriction mapping of the *est* β 2 gene, whether it is identical in these populations of *C. pipiens* to that detected in populations of OP resistant *C. quinquefasciatus* (Raymond *et al.*, 1991). However, if this is found to be the case, it may have important consequences for the control of *C. pipiens*. If the co-elevated Est α 2/Est β 2 based OP resistance mechanism has been transferred from one species to the other, there is no reason to believe why other mechanisms might not similarly be transferred.

Recently, two clonal lines of the tobacco aphid *Myzus nicotianae* were found to have amplified esterase genes (and their flanking regions) with restriction fragment patterns indistinguishable from the amplified esterase E4 and FE4 genes in the closely related *Myzus persicae* (Field *et al.*, 1994). Furthermore, the DNA sequence of an approximately 630 base pair fragment of the *Myzus persicae* E4 and FE4 genes were found to be different to each other but identical to their equivalents in *Myzus nicotianae*. It was suggested that the existence of these apparently identical resistance genes in the two species can best be explained by the transfer of these amplified genes to hybrids of the two species by sexual reproduction and then

subsequently into *Myzus nicotianae* populations (Field *et al.*, 1994).

Whilst the example of the transfer of the amplified E4 and FE4 genes from *Myzus persicae* to *Myzus nicotianae*, and (possibly) the transfer of the co-amplified *est* α 2 and *est* β 2 genes from *C. quinquefasciatus* to *C. pipiens* are exceptional in that they require that the hybrid of the two species to have been fertile, it nevertheless raises the interesting question as to whether such gene transfer, especially genes conferring insecticide resistance, has occurred between other insect species in the past (and has not been detected) or may occur in the future.

6.5. FURTHER WORK

There are still several questions that need to be answered in order to test the hypotheses that *est* β 2 has been amplified only once (Raymond *et al.*, 1991) and that variations in the insecticide interactions of Est α 2 and Est β 2 purified from strains of different geographic origin can be accounted for by the presence of different non-elevated Est α s and Est β s in enzyme preparations and/or different levels of amplification of *est* α 2 and *est* β 2 alleles with concomitant variation in the proportion of non-elevated esterases present (Karunaratne *et al.*, 1995a). These are: 1) Are the amplified *est* α 2 genes identical in all strains of *C. quinquefasciatus*?; 2) How variable are the levels of amplification of the co-amplified esterases?; 3) Are non-elevated Est α s and Est β s co-purified with their elevated counterparts?; and, if so, 4) How much do the insecticide interactions of the non-elevated Est α s and Est β s effect the observed k_a s of the 'purified' Est α 2 and Est β 2 preparations?. The first question

has already been partly answered by the construction in our laboratory of restriction fragment patterns for the *estα2* gene and its flanking regions from several strains of different geographic origin possessing it (S.H.P.P. Karunaratne, personal communication). The restriction fragment patterns were identical. This strengthens the hypothesis that *estα2* and *estβ2* were co-amplified only once. This result should, however, be confirmed by sequencing the *estα2* gene from some of these strains to check that small variations in nucleotide sequence, leading to amino acid substitutions and changes in insecticide affinity, have not been missed during this restriction fragment analysis study. The second question can be examined by probing slot blots of genomic DNA extracted from individual larvae from strains having the co-amplified genes with labelled cDNA probes of *estα2*¹ and *estβ2*¹ and measuring any differences in signal. The third and fourth questions can be investigated by expressing both the non-amplified *estα* and *estβ* genes, and the co-amplified *estα2* and *estβ2* genes, using the baculovirus system. This will facilitate an examination of the insecticide interactions of both elevated and non-elevated esterases in isolation from each other.

Both the Colombia and Trinidad *estβ1* genes differ in the size of their *EcoRI* restriction fragments with the two previously analyzed strains (Raymond *et al.*, 1991; Vaughan *et al.*, 1995). To determine if the Colombia and Trinidad *estβ1* genes differ from those in the TEM-R and MRES strains a full restriction map of the *estβ1* genes should be completed. In addition, to determine whether the *EcoRI* site creating the doublet of bands in the two strains lies within the *estβ1* gene (either in the coding sequence or the non-transcribed introns) or in the flanking regions of

the gene, digested DNA can be hybridised with probes specific to the 5' and 3' ends of the gene. If the *EcoRI* site is internal to the gene, hybridisation with either probe should give only one band. However, if the *EcoRI* is external to the gene the doublet of bands will still be seen.

Whilst *Est* α 2 and *Est* β 2 have always been found co-elevated (Raymond *et al.*, 1991), *Est* α 3 has sometimes been found to be co-elevated with *Est* β 1 (in the Colombia, Trinidad and MRES strains), and sometimes not (in the TEM-R strains). In the Habana strain, *Est* α 3 can segregate from *Est* β 1 and it has now to be determined, by reciprocal backcrosses with the susceptible PelSS strain, whether segregation might also occur in the Colombia, Trinidad and MRES strains. In the PelRR strain, the co-amplified *est* α 2¹ and *est* β 2¹ genes are situated on the same amplicon (Vaughan, 1995). Further work needs to be carried out to determine if this is also the case in other strains possessing these amplified genes and, also whether, in the strains that possess both the amplified *est* β 1 and *est* α 3 genes, these genes are situated on the same amplicon. This can be done by using PCR primers that will anneal to the two esterases and give a product that spans the two genes if they lie on the same amplicon. If this gives a positive result a genomic library of one of the strains of interest will be constructed and screened for plaques that are positive for both amplified *est* α and *est* β genes to confirm that the PCR product lies between the amplified esterases and not between one of the amplified esterases and a non-amplified *est* α or *est* β .

Following the discovery of a partial xanthine dehydrogenase (*XDH*) gene on the

same amplicon as the *est* α 2¹ and *est* β 2¹ in the PeRR strain, it was hypothesised that the presence of this gene, if it is complete and functional, may contribute to an additional benefit to the mosquitoes possessing it, and may account for the displacement by these mosquitoes of others that possess only amplified esterases (amplified *est* β 1 alone or co-amplified *est* α 3 and *est* β 1) (Vaughan, 1995). It has now to be determined if the amplified XDH is functional and if the XDH gene is amplified in other strains having co-amplified *est* α 2/*est* β 2.

Expression of both non-amplified and amplified *Culex* esterase genes using the baculovirus system will facilitate site-directed mutagenesis studies to investigate those amino acid changes that bring about increased insecticide binding affinities in the elevated esterases compared to their non-elevated equivalents. It will also facilitate several other investigations. Firstly, as already mentioned above, it will enable the non-elevated and elevated esterases to be characterised in isolation from each other. Secondly, it will enable these esterases to be crystallized for the elucidation of their three-dimensional structure and compare these with the structures already known for *Torpedo californica* AChE (Sussman *et al.*, 1991) and *Geotrichum candidum* lipase (Schrag & Cygler, 1993). This will further aid our understanding of how the esterases sequester insecticides and, hopefully, aid in the rational design of insecticides to overcome the elevated esterase-based resistance mechanism. Thirdly, the expressed amplified esterases could be used to raise monoclonal antibodies that would be specific to each, which could, in turn, be used in the construction of a field test kit to diagnose their presence in populations of *Culex* mosquitoes. Whilst the development of a field test around monoclonal

antibodies is more involved than use of class specific esterases gene probes, the use of monoclonal antibodies may be more readily applied to the field situation, especially in those countries where molecular laboratories have yet to become widespread or where the cost of the molecular biological approach may prove prohibitive. Finally, if a range of esterases, including other carboxylesterases that cause insecticide resistance by quantitative and/or qualitative changes, and insecticide resistance associated phosphatases, from other insects, can be expressed using the baculovirus system, these can then be used to screen insecticides at the development stage for possible interactions which might lead to resistance against them in the field.

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